Structure of Saxitoxin in Solutions and Stereochemistry of Dihydrosaxitoxins[1]

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Abstract: Proton magnetic resonance measurements of the potent neurotoxin, saxitoxin, at different pHs, established that its imidazole guanidine moiety has a weak basicity ($pK_a = 8.24$), and thus a large population of the molecules do not carry a positive charge on the assumed active site around the physiological pH. Also it was discovered that at a higher pH a significant number of the toxin molecules exist as the keto form whose imidazole guanidine is not protonated. The dihydrosaxitoxin, which was shown to lack activity in the earlier pharmacological experiments, was determined to have the 12α-hydroxyl configuration. The isomeric 12β derivative was prepared by sodium cyanotrihydridoborate reduction of saxitoxin. The side-chain conformation of saxitoxin in solution was found to be the same as in the crystalline form. However, the keto form of saxitoxin and the 12α-hydroxy isomer of dihydrosaxitoxin exist in a different rotamer mixture. The results suggest that the presence of 12β-hydroxyl group has a significant effect on the side-chain conformation.

Introduction

Saxitoxin is the first toxin isolated from the toxic shellfish which causes the so-called paralytic shellfish poisonings.[4] Its presence was later confirmed in a number of organisms.[5] The significance of the compound does not remain simply as an extremely noxious substance responsible for the health hazard, but it is an indispensable tool to study the action of various agents to the neuromuscular system and more basically the structure of excitable membranes. Saxitoxin and another potent marine toxin, tetrodotoxin, are unique for their ability to block the influx of sodium ions through the membranes and to stop effectively the formation of action potentials.[6,7] For that reason, the action mechanism of these toxins has drawn so much attention by neurophysiologists in hope of finding the key to solve the mystery of the excitable membrane in the toxin structures.

The structure of saxitoxin, **1**, was established by X-ray crystallography after more than 20 years of chemical study.[8,9] The crystal structure depicts the presence of a unique hydrated ketone stabilized by the two electron-withdrawing guanidium moieties. There have been several attempts to correlate the structure to the blocking mechanism and consequently to the sodium channel structure itself. This paper is intended to present information pertinent to such studies.

Experimental Section

Saxitoxin used in this study was supplied by the U.S. Food and Drug Administration and was checked for purity by TLC and high-pressure LC. Proton magnetic resonance (^1H NMR) measurements were done with a Bruker HX 270-MHz instrument in FT mode. Saxitoxin spectra were measured with a 0.083 M solution in 100% D_2O in a 5-mm tube. The dihydrosaxitoxin spectra were obtained with 0.01 M solutions in 100% D_2O. The pH adjustments were made with NaOD and DCl solutions by inserting a micro combination electrode (Ingold No. 6030) into the tube, and the values were not corrected for deuterium ions. All chemical shifts are expressed in parts per million from disodium 3-(trimethylsilyl)propanesulfonate (DSS).

The numbering system used in this paper is based upon the one used in previous publications,[10] and hydrogens are designated according to the carbon to which they are attached. The symbols α and β indicate the back and front side of the ring, respectively. The *pro-R* and *pro-S* designation was used to specify the 13-methylene hydrogens to avoid confusion.

Catalytic Reduction of Saxitoxin. Saxitoxin dihydrochloride (5.0 mg) dissolved in glass-distilled water (1 mL) was introduced into a flask containing 5 mg of prereduced platinum oxide in 4 mL of water, and the reaction mixture was stirred for 75 min in H_2 atmosphere. The catalyst was filtered and washed. Filtrate and washings were combined and lyophilized. TLC (Whatman LHP-K high-performance plates and pyridine-ethyl acetate-acetic acid-water, 15:10:3:4)[11] showed, after treatment with Weber spray reagent and heating of the plate at 120 °C for 10 min, one predominant spot (R_f 0.38) and another minor spot (R_f 0.43). Purification of the major component was performed by chromatography using Bio-Rex 70 columns (6 × 500 mm)[12] and elution with 0.25 N acetic acid. Fractions of 2.5 mL each were collected every 15 min. Fractions 23-26 showed minute traces of the R_f 0.43 component while fractions 30-34 contained the R_f 0.38 component which after concentration and lyophilization gave (12*R*)-dihydroxaxitoxin, **2** (3.9 mg).

Sodium Cyanotrihydridoborate Reduction of Saxitoxin. Saxitoxin dihydrochloride (5.1 mg) in 50% aqueous methanol (1 mL) was treated with 2.0 mg of $NaB(CN)H_3$ in 0.5 mL of 50% aqueous methanol at pH 6.2, and the mixture was stirred at room temperature for 3 h. One drop of acetone was added to decompose excess reagent, and the mixture was then frozen and lyophilized. The residue was dissolved in 1 mL of water and passed through a Bio-Rex 70 column (9 × 60 mm). The inorganic ions were removed by washing with 20 mL of glass-distilled water followed by 30 mL of 0.25 N acetic acid to elute the reaction product. The acetic acid eluate was concentrated and lyophilized. The residue (4.2 mg) showed two spots on TLC (R_f 0.38 and 0.43) using the same conditions as in catalytic reduction. Separation was performed on a Bio-Rex 70 (minus 400 mesh) column (6 × 500 mm) using 0.25 N acetic acid, and fractions of 2.5 mL were collected every 15 min. Fractions 22-30 (A) showed one spot (R_f 0.38) on TLC whereas fractions 32-34 (B) showed the other (R_f 0.43). Concentration and lyophilization of the product containing fractions yielded A (2.1 mg), (12*R*)-dihydrosaxitoxin, **2**, and B (2.0 mg), (12*S*)-dihydrosaxitoxin, **3**.

Results and Discussion

(I) pH Dependency of Chemical Shifts. The 270-MHz ^1H spectrum of saxitoxin dihydrochloride dissolved in D_2O (pH 4.8) (Figure 1a) was in agreement with the reported spectra.[13,14]

(1) Most contents of this paper were reported at the Marine Natural Products Symposium, ACS-CJS Joint Congress, Honolulu, 1979, and the Annual Meeting, American Society of Pharmacognosy, West Lafayette, 1979.
(2) Postdoctoral Research Associate 1977-1979.
(3) Postdoctoral Research Associate on leave from Faculty of Pharmacy, Cairo University, 1979-.

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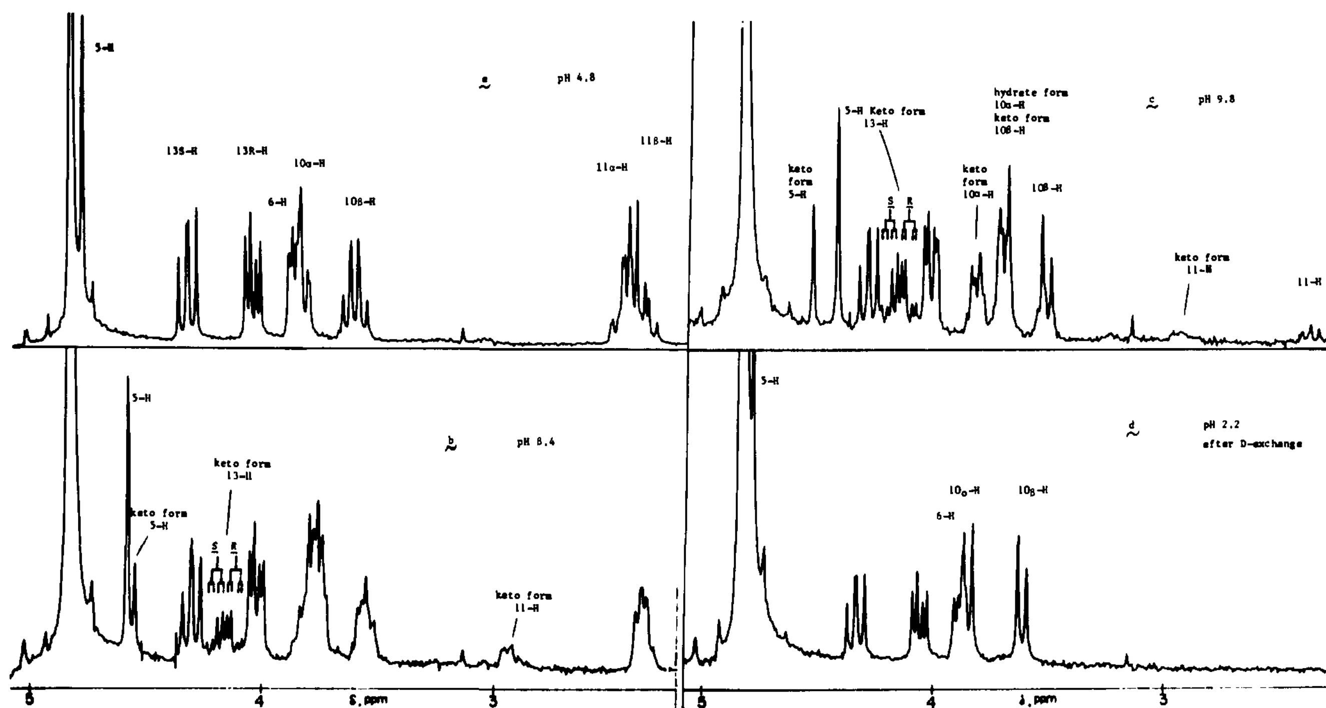

Figure 1. 270-MHz ^1H NMR spectra of saxitoxin at different pHs. The spectra were measured in D_2O at 296 K.

However, when the pH solution was raised to higher pH, changes in chemical shifts were observed with some protons (Figure 2). The change was most prominent with 5-H and to a lesser degree with 6-H and 10-H.

Saxitoxin was known to have two observable pK_a's, 11.5 and 8.24.[15] The largest chemical shift change observed with 5-H precisely coincides with the latter pK_a value. Since it is reasonable to assume that the chemical shifts of protons on a carbon attached to a dissociating moiety will be most influenced by pH change,[16] pK_a = 8.24 was determined to be of the imidazole ring guanidine. Previously we observed the similar pH dependency of chemical shifts with neosaxitoxin in its structure determination.[10] It should be noted that the X-ray crystallography data reported on saxitoxin di-*p*-bromobenzenesulfonate[8] record a longer bond distance, 1.36 Å for C(8)–N(9) compared to 1.32 Å for the other guanidium. This possible implication of less involvement of N-9 in the hybridization, however, was not observed in the crystallography data of saxitoxin hemiketal dihydrochloride which reported all equal bond lengths for the guanidium C–N bonds.[9]

(II) Signals due to the Tautomeric Form of Saxitoxin. The spectrum of saxitoxin at a higher pH showed another manifestation besides the chemical shift changes; a new set of signals due to a tautomeric form appeared in spectra taken at pH above 7.0 (Figure 1b,c). The proportion of the tautomeric component increased as pH was raised to 10.6 where the spectral features changed drastically due to the deprotonation of the second guanidium group. The tautomer was determined to be the keto form of saxitoxin because, in its spectrum, (i) 11-H signal shifted to a lower field (+0.51 ppm) to δ 2.92, an appropriate position for methylene protons adjacent to a carbonyl group (The signal rapidly disappeared due to deuterium exchange through enolization.[17]), (ii) 5-H proton shifted to an upper field (−0.24 ppm) to δ 4.1 as a result of relief from the 1,3-diaxial deshielding effect of 12β-hydroxy group (Table I), (iii) one of the 13-methylene protons shifted to an upper field (−0.13 ppm) and the other slightly to a lower field (+0.06 ppm), reflecting the changes caused by the replacement of the 12β-quasi-axial hydroxy group by a ketone (vide infra), and (iv) no irreversible chemical change took place during the entire NMR measurement as proved by the restoration of the original spectrum after acidification of the test solution except for that of the deuterium exchanged 11-methylene protons (Figure 1d).

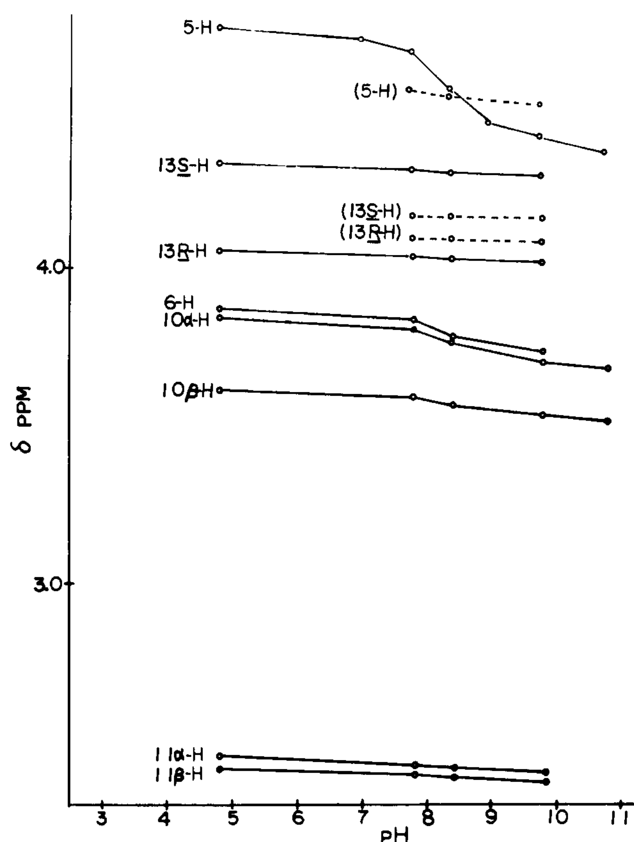

Figure 2. Proton chemical shift changes of saxitoxin at different pHs.

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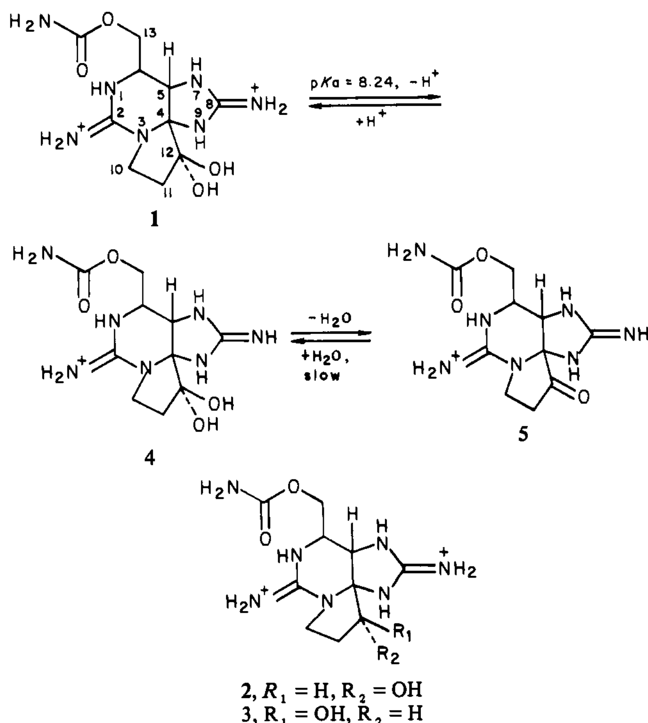
(17) A solution of saxitoxin dihydrochloride is known to undergo a slow exchange of the 11 protons in D_2O_3, which usually takes 1 week or 2 weeks. We discovered, however, that this exchange is tremendously enhanced at elevated pHs, and complete exchange took place within the 1 h required for the NMR experiment.

Table I. Proton Signal Parameters of 270-MHz Spectra of the Two Forms of Saxitoxin

	hydrate form (pH 4.8)		keto form	
proton	chem shifts, ppm	coupling const, Hz	chem shifts, ppm	coupling const, Hz
5-H	4.77	$J_{5,6} = \sim 1$	4.53	$\simeq 0$
6-H	3.87	$J_{6,13S} = 9, J_{6,13R} = 5, J_{5,6} = 1$	4.01	*a*
10α-H	3.83	$J_{10,10\beta} = 9.3, J_{10\alpha,11\beta} = 2.3$	3.80	*a*
10β-H	3.61	$J_{10\alpha,10\beta} = 9.3, J_{10\beta,11\beta} \simeq J_{10\beta,11\alpha} \simeq 10$	3.69	*a*
11-H	2.41		2.92	*a*
13-*pro-S*-H	4.32	$J_{13R,13S} = 11.2, J_{13S,6} = 9.2$	4.19	$J_{13S,6} = 6.2, J_{13S,13R} = 12.0$
13-*pro-R*-H	4.05	$J_{13R,13S} = 11.2, J_{13R,6} = 5.1$	4.11	$J_{13R,6} = 5.2, J_{13R,13S} = 12.0$

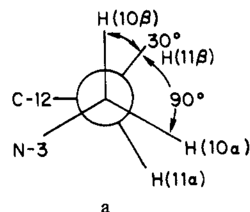
[a] Could not be determined due to overlap with signals from hydrate form.

Scheme I



The rate of interconversion between the keto and hydrate forms of saxitoxin is slow enough to be clearly distinguished spectroscopically. In the pH range used for the measurements, the chemical shift of 5-H and other protons of the keto form saxitoxin remained unchanged, indicating its imidazole guanidine group is already completely deprotonated. In other words, the keto form is allowed to exist only when there is no positive charge on the guanidine.

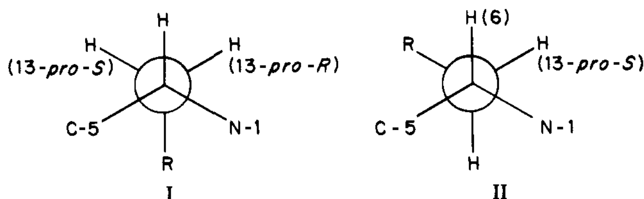
(III) Conformation of Saxitoxin Molecules. The crystal structure of saxitoxin shows the four atoms N-3, C-4, C-10, and C-11 forming a plane and C-12 out of this plane by 0.62 Å.[8] The observed coupling constants, which include a very small coupling between 10α-H and 11β-H, place the dihedral angles on the five-membered ring as shown by **a**. This indicates that the

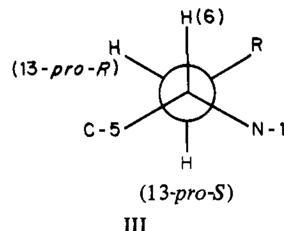


a

conformation of the five-membered ring in solution is identical with that in crystal. The same conclusion has recently been reported by Niccolai et al. in the analysis of the 270-MHz ^{1}H NMR spectrum of saxitoxin.[14]

In order to discuss the side-chain conformation, it was necessary first to assign the 13-geminal proton signals. It was assumed that (i) the lower field proton, δ 4.32, with a larger coupling constant (9.2 Hz) must be the one more frequently facing the (β) side of the molecule because its chemical shift is affected by the OH → C=O conversion at C-12 and (ii) the carbamate side chain extends toward the outside of the molecule as in the crystal form.[8] On the basis of these two assumptions, the signal, δ 4.32, with a coupling constant *J* = 9.2 Hz was assigned to 13-*pro-S*-H(β), and the other (δ 4.05 (*J* = 5 Hz)) to 13-*pro-R*-H(α). If we further assume that the observed coupling constants are solely derived from the arithmetic average of the three rotamers I, II, and III, the solution of eq 1 and 2 gives populations of 18% for I, 22% for II, and 60% for III, which places III as the most populated rotamer in agreement with the crystal data.[8,9,18]





(13-*pro-S*)

III

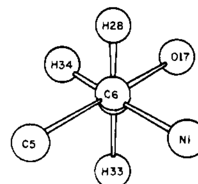
$$J_{\text{obsd}} \text{ for 13-}pro\text{-}S\text{-H} = 9.2 = P_{\text{I}}(2.6) + P_{\text{II}}(2.6) + P_{\text{III}}(13.6) \quad (1)$$

$$J_{\text{obsd}} \text{ for 13-}pro\text{-}R\text{-H} = 5.1 = P_{\text{I}}(2.6) + P_{\text{II}}(13.6) + P_{\text{III}}(2.6) \quad (2)$$

$$P_{\text{I}} + P_{\text{II}} + P_{\text{III}} = 1$$

The keto form saxitoxin, **5**, has different coupling constants, $J_{13\text{-H},6\text{-H}}$ for 13 protons. Similar calculation gave populations of P_{I} = 47%, P_{II} = 29%, and P_{III} = 24%. The result indicates that

(18) Niccolai et al.[14] did the same treatment and reached a different conclusion that II is the most populated species (P_{I} = 0.08, P_{II} = 0.66, P_{III} = 0.26) and also claimed that their result was also in agreement with the X-ray data. The problem seems to be the assignment of the protons. Obviously if one assigns the signal with *J* = 5.1 Hz for eq 1 and the other with *J* = 9.2 Hz for (2), the answer will be reversed. But still it does not account for the discrepancy in the populations. We plotted the coordinate parameters reported by Bordner et al.[9] which is shown in the Newman projection. It is close to III and not II as claimed by Niccolai et al.[14]



This is a computer projection of saxitoxin crystallographic data along the C6–C13 bond. The original numbering system which was used by Bordner et al.[9] and also adopted by Niccolai et al.[14] is shown for comparison. Their C16 corresponds to C13, H28 to 6-H, H33 to 13-*pro-S*-H, and H34 to 13-*pro-R-H* in this text.

Table II. Proton Signal Parameters of Isomeric Dihydrosaxitoxins

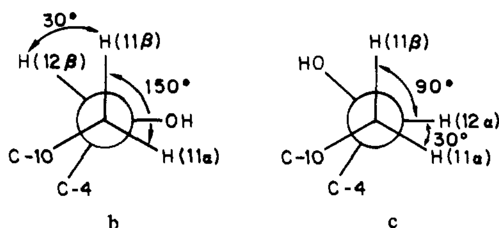
	(12*R*)-dihydrosaxitoxin (2)		(12*S*)-dihydrosaxitoxin (3)	
proton	chem shifts, ppm	coupling const, Hz	chem shifts, ppm	coupling const, Hz
5-H	4.64	$J_{5,6} = 1.5$	4.79	$J_{5,6} = 1.0$
6-H	3.86	$J_{5,6} = 1.5, J_{6,13S} = 4.8, J_{6,13R} = 5.9$	3.85	$J_{5,6} = 1.0, J_{6,13R} = 5.9, J_{6,13S} = 9.5$
10α-H	3.75	$J_{10\alpha,11\beta} = 1.8, J_{10\alpha,10\beta} = 10.2, J_{10\alpha,11\alpha} \simeq 10$	3.79	$J_{10\alpha,11\beta} = 1.8, J_{10\beta,10\alpha} = 9.5, J_{10\alpha,11\alpha} = 8$
10β-H	3.56	$J_{10\alpha,\beta} = 10.2, J_{10\beta,11\alpha} = J_{10\beta,11\beta} \approx 1$	3.77	$J_{10\alpha,10\beta} = 9.5, J_{10\beta,11\alpha} = J_{10\beta,11\beta} = 8$
11α-H	2.02		2.31	m
11β-H	2.49	$(J_{10\alpha,11\beta} = 1.8)$	2.42	m
12α-H			4.35	$J_{11\alpha,12\alpha} = 3.7, J_{12\alpha,11\beta} = 0$
12β-H	4.37	$J_{11\beta,12\beta} = 7.7, J_{11\alpha,12\beta} = 11.0$		
13-*pro-R*-H	4.19	$J_{6,13R} = 5.9, J_{13R,13S} = 11.7$	4.06	$J_{13R,13S} = 11.7, J_{13R,6} = 5.9$
13-*pro-S*-H	4.22	$J_{6,13S} = 4.8, J_{13R,13S} = 11.7$	4.32	$J_{13R,13S} = 11.7, J_{13S,6} = 9.5$

the presence of the quasi-axial 12β-hydroxy group has a considerable influence on the rotation of the side chain.

(IV) Stereochemistry of Dihydrosaxitoxins. The determination of the stereochemistry of dihydrosaxitoxin is an important subject but has been left in an ambiguous manner. Bordner et al. depicted the 12α-hydroxy structure in their paper without explanation.[9] Dihydrosaxitoxin prepared by catalytic hydrogenation according to the reported procedure[19] gave predominantly one isomer, **2**, with only a trace amount of the other isomer, **3**. Chromatography on Bio-Rex 70[12] with acetic acid solution afforded a pure specimen of **2**. Meanwhile, the reduction of **1** with $NaB(CN)H_3$ at pH 6.2 gave a 50:50 mixture of **2** and **3**, which was separated by chromatography.

The 270-MHz ^1H NMR parameters obtained with both **2** and **3** are listed in Table II. The assignment of the protons was accomplished by decoupling experiments.

Since the coupling constants of 10-H and 11-H of both compounds are almost identical with those of saxitoxin, the conformation of the five-membered ring in **2** and **3** seems to be very close to that of saxitoxin (vide supra) even after reduction. Using a model and the Karplus curve, we could readily decide that **2** is the 12α-hydroxy (12*R*) and **3** is the 12β-hydroxy isomer (12*S*). The Newman projections at C-11 and C-12 of **2** and **3** are shown in **b** and **c**, respectively. This assignment was also supported by the fact that 5-H of **2** shifted to an upper field (−0.13 ppm) compared to that of saxitoxin, while 5-H in **3** showed little change (+0.02 ppm). This sizable shift was caused again by the relief from the effect of 12β-OH, which was in 1,3-diaxial relation to 5-H.



The almost exclusive formation of the 12α isomer by catalytic hydrogenation was not unexpected since there was some indication that bulky groups attack saxitoxin preferentially from the β side. The formation of the 12β-ethyl hemiketal or stereoselective exchange of ^{18}O in $H_2{}^{18}O$ at C-12 of saxitoxin is an example.[9,13]

Expectedly isomer **3** with the 12β-quasi-axial hydroxy group shows coupling constants for the 13α and 13β protons almost identical with those of saxitoxin, suggesting that the side chain takes a similar conformation as in saxitoxin hydrate. On the other hand, isomer **2** showed coupling constants very close to those of the keto form saxitoxin, **5** (calculated as a mixture of rotamers: I, 50%; II, 30%, III, 20%). This again demonstrates the large influence of the quasi-axial 12β-hydroxy group on the side-chain rotation.

Table III. Population (%) of Different Forms of Saxitoxin at Different pHs[a]

form (no. of change)	pH 4.8	pH 7.7	pH 8.4	pH 9.8
keto form (+1)	0	1	24	36
hydrated (+1)	0	23	37	62
(+2)	100	76	39	2

[a] The populations of the keto form were obtained from the NMR integration, and the ratios of the total single charged species were calculated from Henderson-Hasselbach equation.

Conclusion

There has been considerable confusion as to the origin of the second pK_a (8.24) of saxitoxin. Because of its anomalous behavior in titration and its low value, this pK_a was first attributed to the dissociation of an –OH group.[13,20] Recently one of the guanidium groups was considered again as the source of this pK_a.[9,21] Nevertheless in the neurophysiological discussions the ketone hydrate is still often assumed as an acidic dissociable group associated with this pK_a. Our results exclude unequivocally such possibility. They also correct another misconception that the imidazole guanidine group, which is generally considered to play the major role in the blockage of the sodium channels, has the pK_a of 11.5 on an assumption that the pyrimidine ring guanidine might have a lower pK_a value because of the steric strain introduced by the fusion of a five-membered ring. To the contrary our results show that the imidazole guanidine has a very weak basicity and that at a higher pH range a significant number of molecules exist in the keto form whose imidazole immidazole guanidine is not protonated at all (Table III). Thus within the pH range of 7.0–8.0, at which most experiments on the membrane action potential are conducted, saxitoxin exists as an equilibrium mixture of the bivalent cationic hydrate, the univalent cationic hydrate and univalent keto form. Typically at pH 7.7, a population of 76% of the hydrated form with a positive charge on the imidazole guanidine, 23% of the hydrated form with no charge on the guanidine, and 1% of keto form with no charge on the guanidine was deduced by calculation and NMR integration. This new finding may drastically alter some of the concepts surrounding the penetration mechanism of the guanidium group into the sodium channels.

Saxitoxin binds rather firmly to the sodium channels. Participation of hydrogen bonding, the anion resulting from the dissociation of the hydrated ketone, and hemiketal (or hemiaminoketal) formation with a functional group on the channels are among the factors often discussed by various workers, and some models were presented.[22,23] The possibility of the anion formation is now clearly excluded. The establishment of the stereochemistry of dihydrosaxitoxin is essential for discussing the

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possible participation of the hydrogen bonding. The fact that dihydrosaxitoxin has less than one-hundredth of the original activity on the excitable membranes[19] has been regarded as important information in this respect. In a way hydrated saxitoxin can be regarded as a concoction of the two isomeric dihydrosaxitoxins. Now that the stereochemistry of dihydrosaxitoxin used in the past was established as the 12α-isomer, the neurophysiological study of the 12β-isomer seems to be the next step. Our finding that the absence of 12β-hydroxyl group drastically changed the sterical environment of the β side of saxitoxin molecule as exemplified by the change in the side-chain conformation may be significant with regard to the manifestation of toxicity.

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Kinetic Applications of Electron Paramagnetic Resonance Spectroscopy. 36. Stereoelectronic Effects in Hydrogen Atom Abstraction from Ethers[1]

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Abstract: Relative rates of hydrogen atom abstraction by photogenerated *tert*-butoxyl from a variety of cyclic and acyclic ethers, acetals, and orthoformates have been measured at −60 °C by an EPR spectroscopic technique. There is a pronounced stereoelectronic effect which produces high rates of abstraction from those C–H bonds adjacent to oxygen which have a relatively small dihedral angle (ca. 30°) with respect to the p-type orbital(s) on the oxygen(s). For C–H bonds which have a large dihedral angle (ca. 90°) abstraction is very much slower. The barrier to inversion of the 2-methoxy-1,3-dioxolan-2-yl radical is ≥10 kcal/mol.

In an outstanding series of papers Deslongchamps et al. have shown that molecular reactivities in many areas of heterolytic chemistry are determined by the relative orientation of the bond being broken or made and lone pairs on heteroatoms attached to the reaction center, i.e., by stereoelectronic factors.[3-6] In contrast, in homolytic chemistry the role that adjacent lone pairs play in determining reactivity has received very little experimental attention, particularly insofar as C–H bond homolysis is concerned.[7] In fact, the only published experimental study is that of Hayday and McKelvey,[10] who found that at ambient temperatures triplet benzophenone abstracted the axial 2-H from *cis*-2-methoxy-4-methyltetrahydropyran at least 8 times faster than it abstracted the equatorial 2-H from the trans isomer. Since both compounds gave the same product distribution, it was concluded that a common free radical was formed. This was subsequently confirmed by Malatesta et al.[11] by using EPR spectroscopy to identify common 2-alkoxytetrahydropyran-2-yl radicals generated from conformationally biased cis and trans precursors. The *tert*-butoxyl radical also exhibits a strong preference (ca. 12-fold at ambient temperatures) for the axial 2-H over the equatorial 2-H in its reactions with conformationally biased pairs of 2-methoxy-1,3-dioxanes and 2-methyl-1,3-dioxanes.[12]

Despite, or perhaps because of, the dearth of experimental data regarding the role of stereoelectronic effects on the strength of C–H bonds adjacent to heteroatoms, the subject has received considerable theoretical attention, particularly by Wolfe et al.[13-16] Thus, for example,[14] in methanol the strengths of the C–H bonds anti and gauche with respect to the O–H bond have been calculated to be ca. 100 and ca. 95 kcal/mol, respectively, a stereoelectronically induced difference of 5 kcal/mol. Wolfe[17] has also emphasized that certain physical properties of C–H bonds which can be related to C–H bond strengths show a strong dependence on orientation when the C–H bond is adjacent to a heteroatom. Examples include C–H bond stretching frequencies in the IR spectra of amines (the Bohlmann bands)[18] and other heterocompounds[13,16] and ^{13}C–^{1}H coupling constants[19] in the NMR spectra of carbohydrates.[20]

We have been interested in studying the structure, conformation,

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