Study of the Stereochemistry, Relaxation Mechanisms, and Internal Motions of Natural Products Utilizing Proton Relaxation Parameters: Solution and Crystal Structures of Saxitoxin

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Abstract: It is demonstrated that spin–lattice and cross-relaxation rates of protons are effective and accurate determinants of stereochemistry and internal motion of saxitoxin and hence of natural products in general. Proton relaxation mechanisms are essentially dipolar. Spin–spin analysis of the saxitoxin spectrum gave scalar coupling constants and chemical shifts for all protons. The ^3J values for H29, H30, H31, and H32 of the five-membered ring gave interproton distances agreeing within 0.1 Å with those found in the crystal, and the principal side-chain rotamer determined from $^3J_{27,33}$ and $^3J_{28,34}$ was that found in the crystal. The double Karplus curve approach used here is a novel approach to *R* and *S* proton assignment and to determining small-ring stereochemistry. All monoselective, biselective, and nonselective spin–lattice relaxation rates were measured; where monoselective measurements yielded values which still involved cross relaxation, these were corrected to yield true monoselective relaxation rates. The correlation time for each geminal proton vector of the saxitoxin framework was 8.3×10^{-11} s, proving the framework to be rigid. Interproton distances, calculated from cross-relaxation rates, σ, agreed within ±0.2 Å of those found in the crystal and from scalar coupling constants.

I. Introduction

Nuclear Overhauser effect studies of natural products[2,3] and biopolymers[4-8] have indicated the importance of dipolar relaxation mechanisms for protons. It has been suggested that dipolar relaxation mechanisms predominated for monosaccharides[9] and nucleotides[10] by measuring proton spin–lattice relaxation rates in the mono-, bi-, and nonselective modes. NOE and selective excitation relaxation studies have been reported for amino acids and peptides;[6,11-14] proton–proton distances and correlation times were evaluated. It is imperative that the potential of these techniques for measuring interproton distances, absolute configurations, and correlation times for complex natural products be tested and explored.

We described here such a study of saxitoxin, a natural product of established crystal structure.[15,16] Specifically, we report (a) evaluation of mono-, bi-, and nonselective proton spin–lattice relaxation rates, *R*; (b) the use of *F* values (ratio of nonselective to monoselective *R* values) and cross-relaxation parameters, σ (difference between biselective and monoselective *R* values), to determine correlation times; (c) the measurement of interproton distance from proton relaxation parameters and scalar coupling constants and comparison of these with crystal interproton distances; (d) that proton relaxation mechanisms are extensively dipolar; (e) a method of determining the complete absolute stereochemistry of small rings.

II. Experimental Section

Lyophilized saxitoxin was dissoved in 100% D_2O and samples were thoroughly degassed in order to remove oxygen. Care was taken to prevent contamination by other paramagnetic impurities. Spectra were recorded on a Bruker WH-270 equipped with an 1180 Nicolet computer and the temperature was controlled at ±1 °C by a Bruker unit. Nonselective, monoselective, and biselective partially relaxed spectra were obtained with a $(180°-\tau-90-T)_n$ pulse sequence; the selective 180° pulse was provided by the decoupler channel. To perform the double selective experiment the decoupler pulse was frequency modulated by a Hewlett-Packard 3300A function generator. The ^1H NMR spectrum of saxitoxin is shown in Figure 1.

III. Results and Discussion

The complex spin–lattice pathway of each proton in a multispin system cannot be solved by a conventional nonselective R_1 experiment. For a nucleus, *i*, in a molecular environment of other nuclei, *j*, the nonselective (NS) spin–lattice relaxation rate is described by

$$R^i(\mathrm{NS}) = \sum_{i \neq j;m} {R_m}^{ij} + \sum_{i \neq j} \sigma^{ij} \tag{1}$$

where *m* accounts for several relaxation contributions including intramolecular (IDD) and intermolecular (XCC) dipole–dipole interactions, scalar coupling (SC), spin rotation (SR), and chemical shift anisotropy (CSA) mechanisms; σ refers to cross relaxation. Protons have a large natural abundance and a large nuclear magnetic moment, and are thought to relax chiefly by an IDD mechanism according to eq 1 where the R^{ij} and σ_{ij} terms are related to the interproton distance H_i–H_j and the correlation times of these vectors.

According to the theory[9]

$$R^{ij} = {W_0}^{ij} + 2{W_1}^{ij} + {W_2}^{ij} \tag{2}$$

$$\sigma^{ij} = {W_2}^{ij} - {W_0}^{ij} \tag{3}$$

The ${W_n}^{ij}$ quantities have been well defined;[17] when $\omega_H{}^2{\tau_c}^{ij\,2} \ll 1$

$$2\sigma^{ij} = R_{ij} = h^2\gamma^4 {d_{ij}}^{-6}{\tau_c}^{ij} \tag{4}$$

and, rewriting eq 1, we have

$$F = R^i(\mathrm{NS})/{R_0}^i(\mathrm{SE}) = 1 + \sum_{i \neq j} \sigma^{ij}/R^i \tag{5}$$

where $R^i = \Sigma_{i \neq j} R^{ij}$. If only IDD is efficient, the former ratio is equal to 1.5 in the extreme narrowing condition.[9] It can be lowered if the SE, SR, and CSA mechanisms contribute to the relaxation of the nucleus, *i*, or because the extreme narrowing conditions do not apply to the system. The initial rates ${R_0}^i$(SE) obtained from monoselective experiments[9] are used like R^i in eq 1, thus permitting experimental evaluation of $\Sigma_{i \neq j}\, \sigma_{ij}/R^i$ terms. The overall structural problem, however, can be solved when each cross term, σ_{ij}, is calculated either from combined NOE and monoselective R^i measurements[7] or when two nuclei are simultaneously excited and ${R_0}^i(\tilde{i},\tilde{j})$ is experimentally obtained; the relationship ${R_0}^i(\tilde{i},\tilde{j}) = {R_0}^i + \sigma_{ij}$ holds and the cross-relaxation term is given by the difference ${R_0}^i(\tilde{i},\tilde{j}) - {R_0}^i$(SE).[19]

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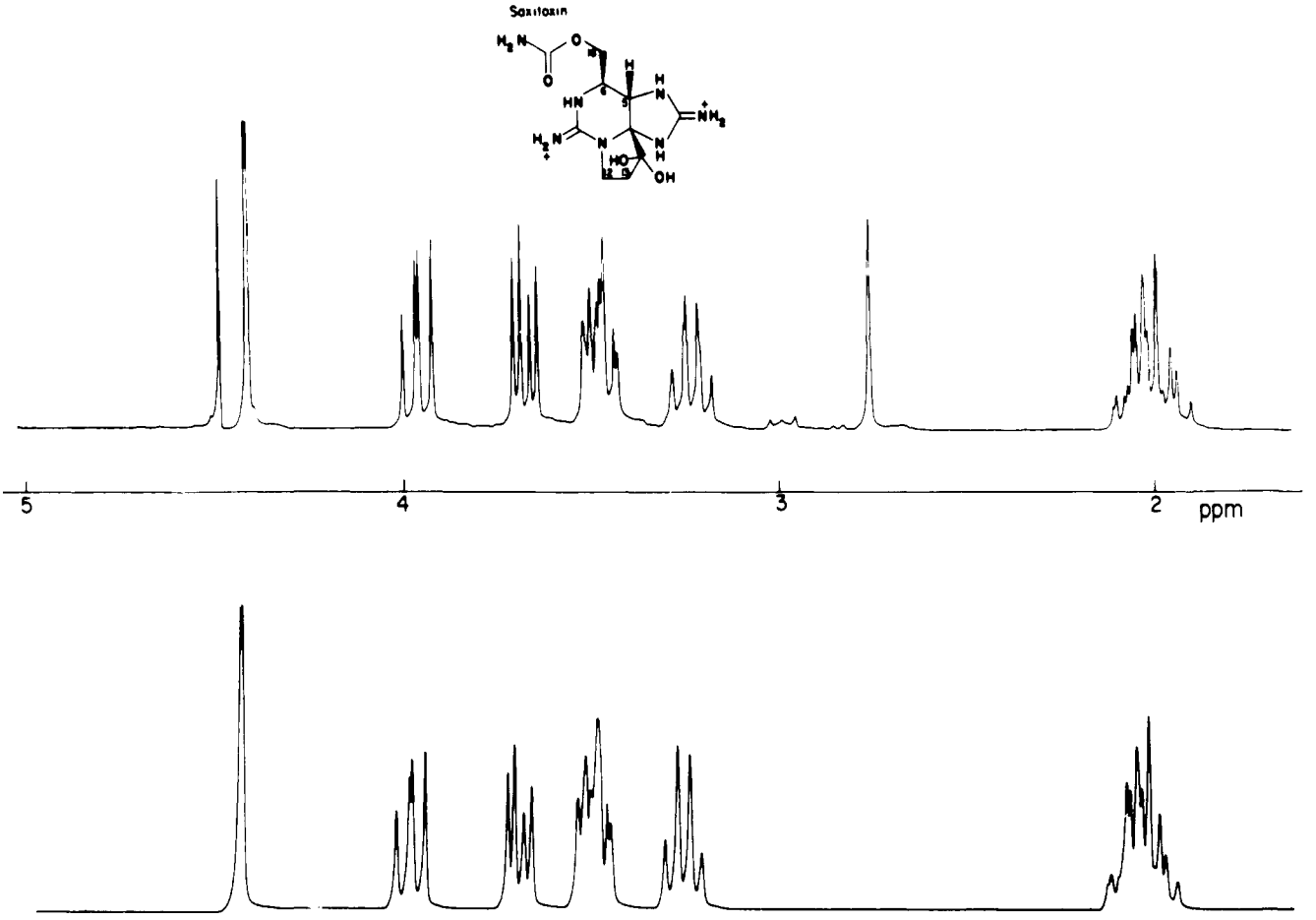


Figure 1. A comparison of the observed (upper) and simulated (lower) 270-MHz NMR spectra of saxitoxin in D_2O. The singlets at 2.76 and 4.49 ppm are acetate and HDO, respectively.

In general, $\sigma_{ij} = h^2\gamma^4 (1/d_{ij}^6) f(\tau_c^{ij})$; to obtain d_{ij}^6 we need to know $f(\tau_c)$. Niccolai et al.[11] have shown that τ_c can be evaluated for geminal distances in amino acids, d_{gem}, since the latter is independent of conformation.[20] In a rigid molecule this same equation and τ_c^{gem} can be used to determine $d_{dihedral}$ or any other relevant interproton distance.[11]

To apply the above to the field of natural products and biopolymer conformation and dynamics, it is important to first of all study rigid molecules of known structure (interproton distances). Saxitoxin is a crystallographically defined molecule,[15,16] and is used here to test (1) the relevance and the role of dipolar relaxation; (2) quantitative use of proton relaxation parameters from the IDD general equations, for interproton distance and correlation time measurement; (3) to define a general method for a self-consistent conformational analysis in solution based upon through-space interactions. The latter fall off rapidly ($1/d^6$) with distance and can be rapidly measured by proton relaxation which complements the approach utilizing scalar coupling constants, the magnitude of which depends on through-bond interactions. The saxitoxin structure and spectrum are shown in Figure 1. The assignment of all the protons agrees with a previous ^1H NMR study,[5,15] and the same nomenclature found in the X-ray paper[16] is used in Table 1.

A. Information from R^i(NS), R^i(SE), and F Ratios. The nonselective and monoselective rates for protons H27–H34 are shown in Table 1A. The F ratios and σ values calculated from these are shown in Tables 1A and 1B, respectively.

The different R(NS) and R(SE) values for the six geminal protons compared with tertiary protons H27 and H28 are explained by the highly efficient relaxation of each geminal proton by its neighbor 1.77 Å away.

In the case of the poorly resolved multiplets for the geminal pair H31 and H32 it is possible to see from the relaxation spectra that the H31 lines collectively relax faster than the high-field H32 lines. Although cross relaxation between H31 and H32 must be considered for total quantitation, their relaxation rates do reflect different relaxation pathways for each proton and hence proton microenvironments. We assumed that the slowest and fastest line of each set approaches the real relaxation rates of these two protons to discuss their spin–lattice relaxation parameters. Furthermore, the fact that in each couple of geminal protons two slightly different relaxation rates are found indicates that the geminal interaction, while predominant, is not the only efficient relaxation pathway.

The F ratios in Table IA give information about (1) mechanisms of proton relaxation and (2) the correlation times for internal and molecular motion.

Protons 28, 29, 30, 33, and 34 have $F \simeq 1.5$ and therefore relax completely by an IDD mechanism; furthermore, the extreme narrowing approximation also applies ($\omega_0^2\tau_c^2 \ll 1$).

H27 has $F = 1.35$ implying that it lies outside the extreme narrowing limit or else relaxation is not 100% dipolar. A more practical explanation can be found because H27 lies close to the H_2O peak and, when it is excited with a 180° pulse to measure its relaxation rate, partial simultaneous irradiation of the H_2O occurs. A small cross relaxation between H27 and H_2O is probably the explanation of why F deviates from 1.5.

Cross relaxation also accounts for the low F ratios, 1.03 and 1.10, for protons H31 and H32. Examination of the spectrum shows that H31 and H32 overlap so much that monoselective excitation by 180° pulse is not easy to achieve.

Table I. ^{1}H NMR Parameters of Saxitoxin

A					
(C*n*)	proton[a] H*i*	chem[b] shift	relaxation rates, s^{-1} [c] R(NS)	R(SE)	R(NS)/R(SE) = F[d]
(C5)	H27	4.41	1.04	0.77	1.35
(C6)	H28	3.50	1.38	0.94	1.46
(C12)	H29	3.24	3.05	2.09	1.46
(C12)	H30	3.48	3.42	2.38	1.44
(C13)	H31	2.07	2.87	2.61	1.10
(C13)	H32	1.97	2.19	2.11	1.03
(C16)	H33	3.96	2.88	2.01	1.43
(C16)	H34	3.68	3.51	2.39	1.46
	HDO	4.61			
	$CHCl_3$	7.27			

B		
scalar coupling constants, Hz	simulated	cross-relaxation rates, s^{-1} R(BS), R(SE)
$^3J_{27,28}$ = 1.4	1.4	$\sigma_{27,28}$ = 0.10
$^3J_{28,31}$ = 9.2	9.2	$\sigma_{28,33}$ = 0.08
$^3J_{28,34}$ = 5.2	5.2	$\sigma_{28,34}$ = 0.08
$^2J_{33,34}$ = 11.5	−11.5	$\sigma_{33,34}$ = 0.61
$^2J_{29,30}$ = 9	8.5	$\sigma_{29,30}$ = 0.65
$^3J_{29,31}$ = 9	8.5	$\sigma_{29,31}$ = 0.14
$^3J_{30,32}$ = 9.2	8.6	$\sigma_{30,32}$ = 0.09
$^3J_{30,34}$ = 2	2	$\sigma_{30,31}$ = 0.14
$^2J_{31,32}$ = 13.5	−13	$\sigma_{31,32}$ = 0.63
		$\sigma_{27,33}$ = 0.04
		$\sigma_{27,34}$ = 0.10

[a] The bound carbon atoms, numbered as in Figure 1, are reported in parentheses for each proton. [b] Chemical shifts are in parts per million from internal $CHCl_3$, assuming δ_{CHCl_3} 7.27 ppm. [c] Relaxation rates evaluated as initial slopes in the semilogarithmic plot of $(A_\infty - A_\tau)/2A_\infty$ vs. τ can be considered within 98% of confidence. [d] F values are affected by a ±5% error.

Finally, because of the high F value for H28 and H30, any cross relaxation between these two protons can be excluded. They are spatially distant in the molecule but spectrally close.

B. σ Values and Correlation Times. It is possible to use σ values plus the geminal dihedral and other interproton distances from the crystal to calculate τ_c for every vector in saxitoxin.[11] This approach will not be taken here, for our objective is to demonstrate that interproton distances and τ_c values can be derived for natural products of unknown structure using proton relaxation parameters and/or scalar coupling constants; therefore, no assumptions about the conformation of saxitoxin will be made.

The geminal proton pair H29 and H30, identified by scalar decoupling, are not strongly coupled ($\Delta\delta/J \sim 9.5$) and their interproton distance, d_{gem}, equals 1.77 Å. This knowledge permitted us to derive from $\sigma_{29,30}$ in Table IB two correlation times, $\tau_{29,30} = 4.0 \times 10^{-10}$ and 8.3×10^{-11} s. Assuming the simplified eq 4, and that saxitoxin fully obeys the extreme narrowing condition, the value $\tau_{29,30} = 7.9 \times 10^{-11}$ s is found; 8.3×10^{-11} s is therefore the correct value. It also agrees with that predicted from the F ratio.

Accurate, direct evaluation of the real $\sigma_{31,32}$ from R(BS) − R(SE) was impossible because their chemical shifts differ by only 0.1 ppm. However, the following approach was effective and should prove generally useful for such cases. R^{31}(SE) and R^{32}(SE) in Table IA must be regarded as biselective relaxation rates if their true monoselective rates R^{31} or R^{32} can be calculated from the following equations.

$$R^{31}(\text{SE}) - R^{31} = \sigma_{31,32}$$

$$R^{32}(\text{SE}) - R^{32} = \sigma_{32,31}$$

Table II. Comparison of Interproton Distances of Saxitoxin Calculated by Three Methods

	crystal	scalar coupling constants, Å	σ parameters
H27–H28	2.60	2.65 (+90°)	2.46
H28–H33	3.06	2.73[a]	2.54
H28–H34	2.50	2.56[a]	2.53
H27–H33	2.80		2.80
H27–H34	2.58		2.44
H29–H32	2.96	3.02 (+150°)	>3[b]
H29–H31	2.32	2.33 (+30°)	2.34
H30–H31	2.68	2.70 (−90°)	2.32
H30–H32	2.30	2.33 (+30°)	2.49

[a] Averaged values as discussed in the text. [b] This interproton distance, as the others not reported here, arises from a σ value equal to zero or within the experimental error, which corresponds to a minimum interproton approach of 3 Å, at the observed correlation time.

Provided that R^{31} and R^{32} are known, $\sigma_{31,32}$ and $\sigma_{32,31}$ can be estimated. To estimate R^{31} and R^{32} it is assumed that the IDD mechanism does hold and therefore

$$R^{31}(\text{NS})/R^{31} = R^{32}(\text{NS})/R^{32} = 1.46$$

where 1.46 is the average F experimentally found for all the other saxitoxin protons. In this manner R^{31} and R^{32} were found to be 1.96 and 1.49 s^{-1} and hence $\sigma_{31,32} = 0.64$ and $\sigma_{32,31} = 0.61$.

It is encouraging that within experimental error $\sigma_{32,31} = \sigma_{31,32}$, thus validating the above assumptions and procedures. Furthermore, the calculated $\sigma_{31,32}$ is similar to the $\sigma_{29,30}$ which was directly and accurately measured by experiment. This means that the two geminal interproton vectors (H31, H32) and (H29, H30) have the same correlation time.

The fact that $\sigma_{33,34}$ for the side-chain geminal protons H33 and H34 equals the other geminal cross relaxation suggests a rigid framework for the whole saxitoxin molecule, with an overall isotropic tumbling described by the correlation time calculated from the H29–H30 vector or a rigid molecule whose side chain has a slow internal reorientation along the C_6–C_{16} axis. A rotamer population analysis from the scalar coupling constants of saxitoxin (see section IIID of this work) will remove this ambiguity.

C. Interproton Distances. Since the correlation time for the saxitoxin molecule lies in the extreme narrowing limit, we can assume that $\tau_c = 8.3 \times 10^{-11}$ s, calculated for the H29–H30 vector, is that for the whole molecule and use this τ_c to calculate all other saxitoxin interproton distances from the appropriate σ terms in Table II. All crystallographic interproton distances were compared with those derived from σ values.

To obtain $d_{29,31}$, $d_{29,32}$, $d_{30,31}$, and $d_{30,32}$ simultaneous excitation of H29 or H30 with H31 and H32 was necessary. Because H31 and H32 have similar chemical shifts (2.07 and 1.97 ppm), mono- and biselective experiments involving either were influenced by the biselective excitation of the other. Despite this, three of the distances calculated agreed within ±0.2 Å of the crystal values ($d_{29,31}$, $d_{29,32}$, and $d_{30,32}$) while one ($d_{30,31}$) agreed within ±0.3 Å. They agreed also with the corresponding distances calculated from scalar coupling constants.

The cross-relaxation data therefore gave distances consistent with the essential similarity of the saxitoxin framework in solution and in the crystalline state; the correlation time for the rigid framework is 8.3×10^{-11} s.

Two other conclusions can be drawn from this data: (1) the protons of the framework relax by a dipolar mechanism and (2) it should be possible to use this relaxation rate approach to evaluate stereochemistry and internal motion of non-crystallographically-defined natural products with confidence.

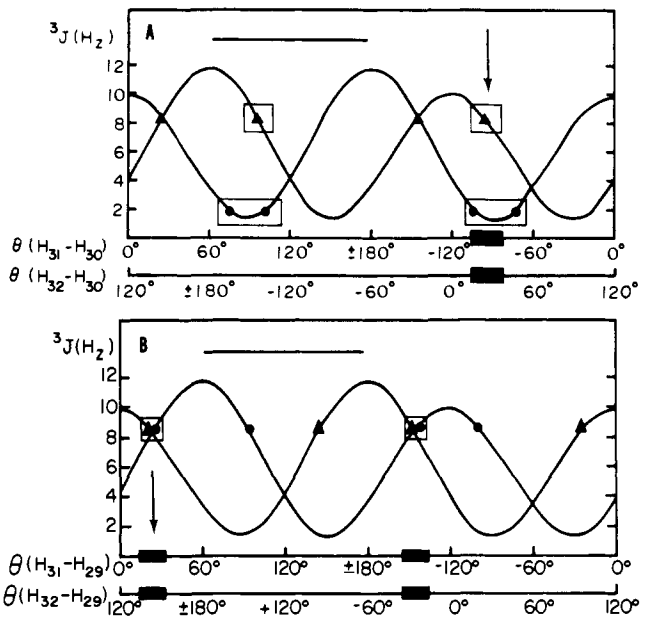


Figure 2. The Karplus curves for the vicinal dihedral angles, θ, and the scalar coupling constants for the proton pairs (H31, H30), (H32, H30), (H32, H29), and (H31, H29). The *R* and *S* proton assignments are those derived by crystallography. The solid bar above the Karplus curves corresponds to θ angles which do not permit closure of the five-membered ring. The boxes on each Karplus curve give the θ angles corresponding to the experimental ^3J values. The solid boxes on the θ axes are those θ angles consistent with both ^3J values between a given proton and both protons which are dihedral to it.

The interproton distances H27–H33, H27–H34, H28–H33, and H28–H34 seem at first glance to agree with the corresponding crystal distances but, as will be seen in the next section, this is not true, and points out the danger of using only one methodology, proton relaxation, to study stereochemistry. It is important that scalar coupling constants (through-bond interactions) and through-space interactions give self-consistent results.

D. Information from Scalar Coupling Constants. Since scalar coupling constants and relaxation parameters arise from complementary through-bond and and through-space interactions, respectively, it is important to compare conformational information derived from both with that predicted from the known crystal conformation. The scalar coupling constants, dihedral angles, and interproton distances derived from them for saxitoxin are shown in Tables I and II; a suitable Karplus curve was used for this.

The geometry of the five-membered ring containing protons 29, 30, 31, and 32 was readily deduced (Figure 3) from Figure 2A. $^3J_{30,31}$ and $^3J_{30,32}$ are each consistent with four dihedral angles. Since $\theta_{30\text{-}31} = \theta_{30\text{-}32} + 120°$, two pairs of dihedral angles, $\theta_{30\text{-}31} = +30$ and $-150°$, are rejected. One of the two angles $\theta_{30,31} = +90$ and $-90°$ can be rejected since it does not correspond to closure of the five-membered ring. Thus $\theta_{30,31} = -90°$ and $\theta_{30,32} = +30°$.

A similar analysis and appropriate Karplus curve (Figure 2B) gave $\theta_{29,31} = +30°$ and $\theta_{29,32} = +150°$.

These dihedral angles yielded interproton distances in excellent agreement with the crystal structure distances (Table II). The ring is therefore rigid in agreement with the correlation time measurements.

The scalar coupling constants, and interproton distances derived from them, for protons (27, 28) and (28, 33, and 34), assuming a rigid system, are shown in Table II. The distance $d_{27,28} = 2.70$ Å, not surprisingly since they are attached to the rigid framework, agrees with the crystal data.

The crystal structure distances for $d_{28,33} = 3.07$ Å and $d_{28,34} = 2.49$ Å correspond to the trans-gauche rotamer II.

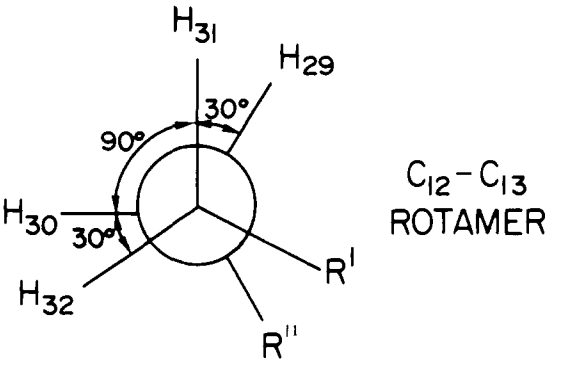
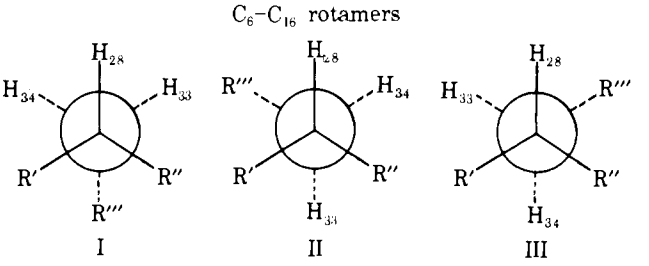


Figure 3. The rotamer corresponding to the measured dihedral angles for the C12–C13 bond of the five-membered ring determined from Figure 2. The arrows on Figure 2 give the correct θ values for each pair of protons.



Rotamer analysis according to eq 6 and 7 yields the following rotamer populations: $p_I = 0.08$, $p_{II} = 0.66$, and $p_{III} = 0.26$.

$$\langle {}^3J_{28,33}\rangle = 9.2 = p_I(2.6) + p_{II}(13.6) + p_{III}(2.6) \quad (6)$$

$$\langle {}^3J_{28,34}\rangle = 5.0 = p_I(2.6) + p_{II}(2.6) + p_{III}(13.6) \quad (7)$$

$$p_I + p_{II} + p_{III} = 1$$

Obviously therefore the most populated rotamer corresponds to that found in the crystal.

A similar rotamer analysis was performed using the observed $\langle\sigma_{28,33}\rangle$ and $\langle\sigma_{28,34}\rangle$ using the three values of p_I, p_{II}, and p_{III}. Because the correlation times for (H28, H33) and (H28, H34) are the same and the gauche-gauche and trans-gauche distances in the rotamers I, II, and III are 2.49 and 3.07 Å, respectively, we could write

$$\langle d_{33,28}^{1/6}\rangle = p_I\left(\frac{1}{2.49}6\right) + p_{II}\left(\frac{1}{3.07}6\right) + p_{III}\left(\frac{1}{2.49}6\right) \quad (8)$$

$$\langle d_{34,28}^{1/6}\rangle = p_I\left(\frac{1}{2.49}6\right) + p_{II}\left(\frac{1}{2.49}6\right) + p_{III}\left(\frac{1}{3.07}6\right) \quad (9)$$

These gave the $\langle d_{34,28}\rangle = 2.76$ Å and $\langle d_{33,28}\rangle = 2.54$ Å in satisfactory agreement (±0.2 Å) with those obtained from the observed, averaged, $\langle\sigma_{28,33}\rangle$ and $\langle\sigma_{28,34}\rangle$, namely, 2.54 and 2.53 Å.

IV. Summary

To establish that proton relaxation rates can accurately delineate solution conformation and internal motions of natural products of unknown structure the mono-, bi-, and nonselective relaxation rates of each proton of saxitoxin were evaluated. From these, interproton distances and correlation times were evaluated assuming that the mechanisms of proton relaxation were exclusively dipolar.

The scalar coupling constants were evaluated by total spin–spin analysis and used to estimate (a) all interproton distances for the five-membered ring and (b) rotamer populations for internal rotation of the side chain of saxitoxin.

The interproton distances for protons H29, H30, H31, H32, H27, and H28 of the molecular framework determined from

spin–spin analysis and cross-relaxation rates agreed with each other (±0.2 Å) and with the crystal structure distances. This proved that the dipolar formalism was applicable to complex natural products and hence proton relaxation parameters can be effectively used to determine the conformation of natural products. The seeming discrepancies between the H30–H31 distance determination from relaxation rate were attributed to experimental and theoretical complications due to non-first-order coupling of H31 to H32. The framework of saxitoxin, as distinct from the side chain, is rigid and the correlation time for overall rotation is $\tau_c = 8.3 \times 10^{-11}$ s.

Internal rotation of the side chain was evaluated from averaged $^3J_{28,33}$ and $^3J_{28,34}$ as well as the observed relaxation parameters $\sigma_{28,33}$ and $\sigma_{28,34}$. Rotamer populations, derived from the scalar coupling constants, and used with similar equations, predicted the average distances $d_{28,33}$ and $d_{28,34}$ from the averaged σ values.

Thus, combined use of the proton relaxation parameters and scalar coupling constants not only completely defined the total absolute stereochemistry of saxitoxin in solution but also gave details of the overall and internal rotations of the molecule. The speed, accuracy, and low concentrations of noncrystalline materials required indicate extensive future use of dipolar coupling in the area of natural-product stereochemistry.

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Proton Spin–Lattice Relaxation Studies of [D-Ala²-Met⁵]Enkephalin

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Abstract: Application of selective and nonselective proton relaxation rate measurements to molecules outside the $\omega_0^2\tau_c^2 \ll 1$ limit is explored using [D-Ala²-Met⁵]enkephalin. Monoselective, biselective, and nonselective measurements yielded cross-relaxation rates, σ, and F ratios; from these, it was deduced that enkephalin has a relatively rigid backbone, internal motion of the Ala², Phe⁴, and Met⁵ side chains, a small reorientation of the Tyr¹ aromatic ring, and proximity of the Ala² and Met⁵ methyl groups. These data support but do not prove the existence of the β-turn conformations. All proton relaxation is dominated by dipolar mechanisms.

Introduction

This study presents novel approaches to the use of mono-, bi-, and nonselective spin–lattice relaxation times for peptides with correlation times outside the extreme narrowing limit.

In the previous papers in this series, we reported proton spin–lattice relaxation rate studies of saxitoxin,[2] whose crystal structure was known,[3,4] and isoleucine.[5,6] These established the mechanisms of proton relaxation and demonstrated the measurement of correlation times and distances for interproton vectors, but the question of larger molecules which do not satisfy the extreme narrowing condition still remained; large natural products and biopolymers in general require spectrometers of high frequency and have long correlation times.

Because of topical interest in enkephalins as a new class of endogenous neurotransmitter peptides[7] and to test the eventual possibility of applying these methods to larger polypeptides and proteins, we report studies of [D-Ala²-Met⁵]enkephalin.

NMR studies of zwitterionic and cationic enkephalins have appeared.[8-12] ^{13}C T_1 data have been interpreted in terms of motion[11,12] and proton spectral parameters, other than relaxation times, have been used to propose various conformations for the zwitterion.[10] The proton relaxation studies of

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