

## NMR Relaxation Studies on Testosterone in Solution. Computer-assisted Analysis of $^{13}\text{C}$ $T_1$ Data Measured at Different Temperatures

Hideaki Fujiwara,\* Yong-Zhong Da, Douwan Zheng,† and Yoshio Sasaki

*Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan*

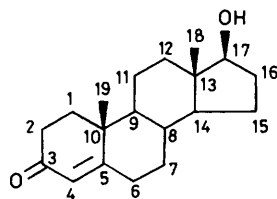
Yoshio Takai and Masami Sawada

*The Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki 567, Japan*

NMR  $^{13}\text{C}$  relaxation times have been measured for testosterone in  $[\text{}^2\text{H}_6]\text{DMSO}$  at various temperatures. The data were processed by a computer-assisted method of analysis based on several molecular reorientational motion models. As a result of this study, activation energies have been determined for the molecular motion. From an analysis of the temperature-dependent  $^{13}\text{C}$   $T_1$  of quaternary carbon atoms, it is seen that the spin-rotation and chemical-shift-anisotropy mechanisms contribute significantly to the quaternary  $\text{sp}^2$  carbons whereas only the spin-rotation mechanism contributes to the quaternary  $\text{sp}^3$  carbons. The experimental relaxation times differ appreciably for the two methyl groups, indicating a difference in the barriers to internal rotation.

The NMR spectra of steroids have recently become tractable because of developments in experimental techniques such as high-field and two-dimensional NMR.<sup>1</sup> These techniques make possible the determination of solution conformations as well as an assignment of the spectra and an identification of the compounds. In addition, NMR spectroscopy can provide valuable information on dynamic solution properties by means of an analysis of relaxation times. For example, Quinn<sup>2</sup> has studied the anisotropic motion of cholesteryl esters by measuring the line widths, relaxation times, and NOE factors of the C-3 and C-6 carbons. Levy *et al.*<sup>3</sup> have analysed the  $^{13}\text{C}$   $T_1$  data for proton-bearing carbons in some steroids based on a fully anisotropic model. They also confirmed the full NOE factors for these carbons at 90.56 MHz. However, such relaxation studies are scarce compared with studies based essentially on chemical shifts and coupling constants. In particular relaxation data are lacking for quaternary carbons, probably because the relaxation time is longer by a few orders of magnitude for these carbons, necessitating a very long measurement time and because the processing of detailed structural data is needed for the calculation of these relaxation times. As regards this problem, Wehli<sup>4</sup> has reported a possible use of relaxation times in the assignment of quaternary carbons.

In the present study, quantitative treatments of the  $^{13}\text{C}$  relaxation times, including quaternary carbons, have been applied to testosterone [ $17\beta$ -hydroxyandrost-4-en-3-one (1)] at various temperatures, and a method of observing



(1) Testosterone.

relaxation times has been pursued. This molecule itself acts as an androgen and is also a key compound in steroid chemistry. The activation energies for the molecular reorientational motion are derived together with the barriers to internal rotation of the methyl groups, and the relaxation mechanisms are discussed for the quaternary carbons.

### Experimental

Testosterone from Tokyo Kasei Co. and  $[\text{}^2\text{H}_6]\text{DMSO}$  from Merck were used without further purification. The sample solution ( $0.48 \text{ mol dm}^{-3}$  in  $[\text{}^2\text{H}_6]\text{DMSO}$ ) was sealed in a  $10\phi$  Pyrex tube after having been degassed by the conventional freeze-pump-thaw method. The  $^{13}\text{C}$  NMR measurements were made on a JEOL PS-100 spectrometer operating at 25 MHz. The temperature of the sample was controlled by an equipped variable-temperature unit, and was monitored occasionally by a  $50 \Omega$  platinum thermometer immersed in a separate tube. The temperature fluctuation was within  $\pm 1^\circ\text{C}$ .

The relaxation time  $T_1$  was measured by an inversion-recovery method. The proton-bearing carbons and the quaternary carbons were measured separately by adopting different waiting times ( $> 5T_1$ ) and different sets of delay times between  $180^\circ$  and  $90^\circ$  pulses. The  $90^\circ$  pulse was  $14 \mu\text{s}$ . The FID signal was averaged 128 times in 8 K points and Fourier-transformed to obtain spectra over an 200 ppm range. The experiment was repeated five times so as to obtain the average and the standard deviation of the relaxation time. The NOE factors were measured at  $40^\circ\text{C}$ , adopting a pulse waiting-time  $> 10T_1$  (120 s). The computer program TIANSOC<sup>5</sup> was used for the analysis of  $^{13}\text{C}$   $T_1$  data. The geometric data were cited from a crystal analysis.<sup>6,†</sup> Proton locations were, however, determined by assigning a standard distance ( $1.09 \text{ \AA}$ ) to the C-H bond length instead of following the relatively inaccurate raw crystal data. Calculations were performed on NEAC S-1000 and SX-1 computers at the Computation Centre, Osaka University.

### Results

The  $T_1$  data for proton-bearing carbons were simulated with the program TIANSOC<sup>7</sup> and the results are listed in Table 1. The molecular dynamics parameters obtained are listed in Table 2. The dynamics models adopted were the isotropic and axially symmetric top models. The fully anisotropic model was not tested thoroughly because a successful fit was not reached in the preliminary calculation. This is probably due to the fact that this

† On leave from: Analytical Centre, Yanbian University, Jilin, China.

‡ These data indicate that the A-ring conformation is in the 'normal' form (ref. 7).

**Table 1.**  $^{13}\text{C}$   $T_1$  Analysis of testosterone in  $[\text{}^2\text{H}_6]\text{DMSO}$  at 25 MHz.<sup>a</sup>

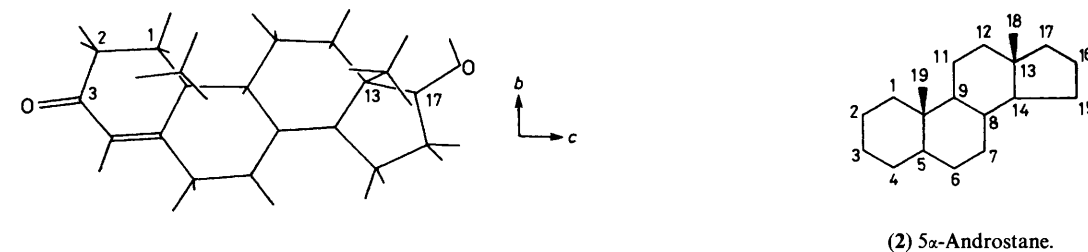
$\delta$ (ppm)	Site	40 °C			70 °C			100 °C		
		$T_{1,\text{obs}}$	$T_{1,\text{calc}}^{\text{DD}}$		$T_{1,\text{obs}}$	$T_{1,\text{calc}}^{\text{DD}}$		$T_{1,\text{obs}}$	$T_{1,\text{calc}}^{\text{DD}}$	
			Model 1	Model 2		Model 1	Model 2		Model 1	Model 2
196.78	3(*)	11.66 ± 0.26	16.63	14.13	19.24 ± 1.36	32.28	27.29	29.10 ± 2.40	55.24	45.88
171.99	5(*)	8.42 ± 0.24	12.30	9.11	14.68 ± 0.28	23.87	17.98	23.37 ± 0.60	40.84	31.13
122.57	4	0.94 ± 0.02	0.94	1.07	1.89 ± 0.01	1.82	2.07	3.19 ± 0.09	3.11	3.62
79.52	17	0.82 ± 0.04	0.87	0.80	1.67 ± 0.02	1.70	1.57	2.77 ± 0.07	2.90	2.62
53.22	9	0.83 ± 0.02	0.88	0.80	1.67 ± 0.02	1.71	1.58	2.75 ± 0.05	2.93	2.66
49.86	14	0.81 ± 0.03	0.88	0.79	1.65 ± 0.02	1.71	1.55	2.68 ± 0.10	2.93	2.60
42.08	13(*)	6.57 ± 0.23	7.62	6.69	11.30 ± 0.31	14.79	12.84	17.93 ± 0.53	25.30	21.85
37.85	10(*)	7.18 ± 0.25	8.73	7.52	12.66 ± 0.24	16.94	14.37	18.74 ± 0.71	28.98	24.17
36.10	12	0.49 ± 0.01	0.47	0.49	0.91 ± 0.02	0.92	0.96	1.62 ± 0.08	1.57	1.65
34.93	1	0.62 ± 0.01	0.47	0.56	1.26 ± 0.02	0.92	1.04	1.94 ± 0.13	1.57	1.73
34.93	8	0.62 ± 0.01	0.87	0.79	1.26 ± 0.02	1.69	1.55	1.94 ± 0.13	2.89	2.59
33.28	2	0.47 ± 0.01	0.47	0.47	0.93 ± 0.01	0.92	0.91	1.63 ± 0.04	1.58	1.61
31.72	6	0.46 ± 0.01	0.47	0.47	0.90 ± 0.02	0.92	0.91	1.53 ± 0.05	1.57	1.58
31.09	7	0.45 ± 0.02	0.47	0.43	0.88 ± 0.06	0.91	0.85	1.52 ± 0.02	1.56	1.49
29.63	16	0.50 ± 0.02	0.47	0.51	0.92 ± 0.04	0.91	0.97	1.60 ± 0.04	1.56	1.67
22.68	15	0.48 ± 0.01	0.47	0.48	0.86 ± 0.01	0.91	0.91	1.53 ± 0.04	1.56	1.49
20.05	11	0.44 ± 0.02	0.47	0.43	0.88 ± 0.02	0.91	0.85	1.55 ± 0.02	1.56	1.49
16.79	19(**)	1.51 ± 0.04	1.51	1.51	2.45 ± 0.06	2.45	2.45	3.67 ± 0.18	3.67	3.67
10.81	18(**)	2.19 ± 0.08	2.19	2.19	3.93 ± 0.20	3.93	3.93	5.74 ± 0.11	5.74	5.74

<sup>a</sup>  $T_1$ /s. Model 1: isotropic model; Model 2: axially symmetric model. (\*): Quaternary carbon, for which  $T_1^{\text{DD}}$  is estimated from the data for proton-bearing carbons. (\*\*): Methyl carbon, for which the rate constant of the internal rotation is determined so as to reproduce the observed value of  $T_1$ .

**Table 2.** Molecular dynamics parameters for testosterone in  $[\text{}^2\text{H}_6]\text{DMSO}$ .

Model	Parameters <sup>a</sup>	40 °C	70 °C	100 °C
Isotropic	$D/10^9 \text{ s}^{-1}$	3.81 ± 0.38	7.39 ± 0.73	12.64 ± 1.25
	S.D./s	0.09	0.16	0.31
Axially symmetric	$D_2/10^9 \text{ s}^{-1}$	1.06 ± 0.07	2.16 ± 0.15	3.69 ± 0.27
	$D_1/D_2$	14.7 ± 2.0	13.2 ± 2.0	13.1 ± 2.1
		28 ± 14	26 ± 18	20 ± 14
		-42 ± 12	-46 ± 19	-53 ± 33
	S.D./s	0.08	0.25	0.28

<sup>a</sup> S.D. = standard deviation between the observed and recalculated  $T_1$  values.



**Figure 1.** Testosterone and its inertial axis system. The principal values of the moment of inertia are:  $I_a = 3\,303.7$ ,  $I_b = 2\,996.9$ , and  $I_c = 617.8 \text{ amu}(\text{\AA})^2$ .

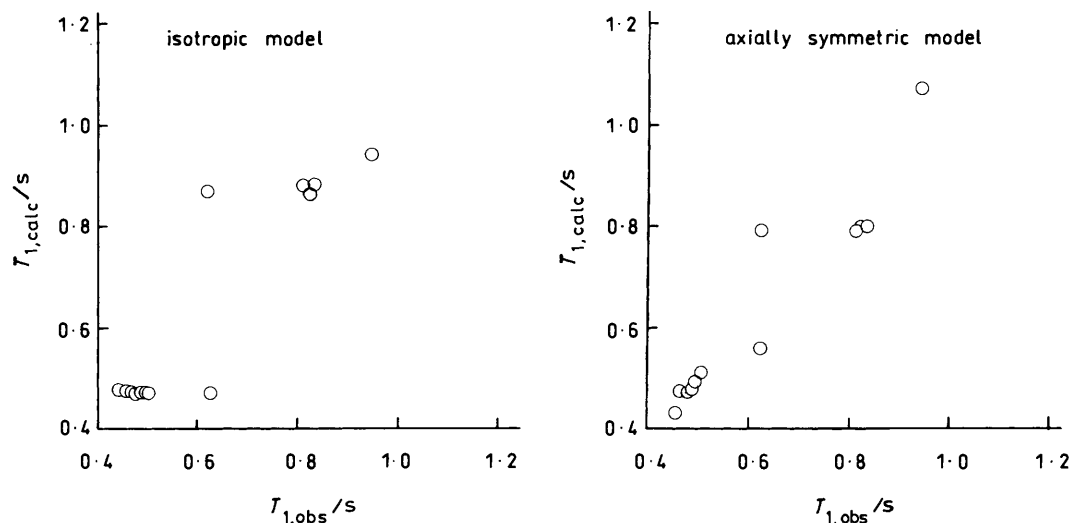
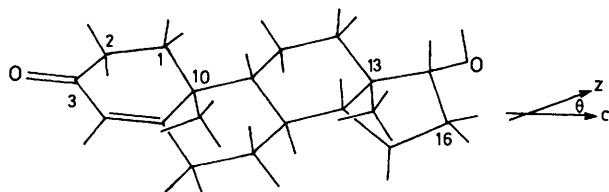
model includes as many as six variables to be optimized but that the raw  $T_1$  data lack sufficient independent data because of the similarity in the directional cosines of the C-H vectors with respect to the main axis of symmetry (see Figure 1 and ref. 2). The anisotropy in the reorientational motion is, however,

thought to be well described by the relatively simple axially symmetric model. For the similar compound 5 $\alpha$ -androstane (2),<sup>3</sup> a fully anisotropic model is adopted, but the reorientational axes are assumed to coincide with the inertial axes, reducing the number of optimized parameters from six to three. The NOE factors were assumed to be full for the proton-bearing carbons, as usually accepted. In fact, the validity of this assumption was confirmed by a preliminary experiment, which gave the values 1.7–2.1 ( $\pm 0.2$ ) at 40 °C. The  $T_1$  data for quaternary carbons were excluded from the simulation. Instead, the dipole-dipole relaxation times were estimated for these carbons using the dynamics parameters obtained above. These values,  $T_{1,\text{calc}}^{\text{DD}}$ , are also included in Table 1 for the quaternary carbons. For these carbons, the NOE factors are easily estimated to be  $1.99(T_{1,\text{obs}}/T_{1,\text{calc}}^{\text{DD}})$ . These values are 1.7–1.9 at 40 °C, supporting the overwhelming contribution of the dipole-dipole relaxation for the quaternary carbons. Such an

**Table 3.** Molecular dynamics parameters for the internal rotation of methyl groups in testosterone.<sup>a</sup>

Site	40 °C						70 °C						100 °C					
	Model 1			Model 2			Model 1			Model 2			Model 1			Model 2		
	$T_{1,obs}$	$D_i$	$V_0$	$D_i$	$V_0$		$T_{1,obs}$	$D_i$	$V_0$	$D_i$	$V_0$		$T_{1,obs}$	$D_i$	$V_0$	$D_i$	$V_0$	
18	2.19	2.54	9.2	3.45	8.4		3.93	3.50	9.3	4.68	8.5		5.74	4.29	9.6	5.02	9.1	
19	1.51	0.95	11.8	0.95	11.8		2.45	1.26	12.2	1.25	12.2		3.67	1.64	12.6	1.66	12.6	

<sup>a</sup>  $T_1$ ,  $D_i$ , and  $V_0$  in s,  $10^{11}$  s<sup>-1</sup>, and kJ mol<sup>-1</sup>, respectively. Model 1: isotropic model; Model 2: axially symmetric model.

**Figure 2.** Comparison of the calculated relaxation times with the observed values for the proton-bearing carbons of testosterone.**Figure 3.** Deviation of the longer axis ( $z$ ) of the molecular reorientational motion from that ( $c$ ) of the moment of inertia.

estimation was also confirmed by NOE measurements. The  $T_1$  values for the methyl carbons were also analysed by the program TIAN SOC which takes into account the internal rotation.<sup>5</sup> In the axially symmetric model, the methyl group is assumed to rotate around an axis tilted from the longer axis ( $z$ -axis, close to the  $c$ -axis) by a definite angle. In practice, the  $z$ -axis and the rate constants,  $D_1$  and  $D_2$ , are fixed (as determined above) so that the  $T_1$  data for the proton-bearing carbons fit well with the experimental data, and the axis of rotation is fixed along the bond between the methyl carbon and the carbon attached to it. The rate constant for the internal rotation  $D_i$  is then simulated in order to reproduce the experimental  $T_1$  value. The calculated  $T_1$  value thus obtained coincides with that observed, since a single experimental  $T_1$  value is used to determine the single parameter  $D_i$ . Here, a sixfold jump model was assumed. The barrier to internal rotation  $V_0$  is then deduced from  $D_i$  according to the relation  $V_0 = -RT \ln(D_i/D_{i0})$ , where  $D_{i0}$  is the rate constant for the zero barrier and is  $0.86 \times 10^{13}$  s<sup>-1</sup> for a methyl group at 30 °C adopting  $(kT/I)^3$  as its measure.<sup>5</sup> The values of  $D_i$  and  $V_0$  thus determined are summarized in Table 3.

## Discussion

### Anisotropic Nature of the Molecular Reorientational Motion.

The calculated values of  $T_1$  are compared with the experimental values in Figure 2 for the proton-bearing carbons. It is clear from Figure 2 that the  $T_1$  values are better reproduced by the axially symmetric model. The calculations show that the anisotropy in the molecular reorientational motion of testosterone is large, *i.e.*, the ratio of the rate constants,  $D_1/D_2$ , is  $>10$  in the axially symmetric model (Table 2). This result resembles that reported for the similar cholesteryl esters,<sup>2</sup> for which the ratio is as great as 40–130. For the latter case, however, the major axis is fixed along an axis connecting the C-3 and C-13 atoms;<sup>2</sup> it is also varied in the calculation in the present case. The main  $c$ -axis for the moment of inertia is close to the line joining the C-3 and C-13 atoms (Figure 1), and the major  $z$ -axis for the molecular reorientational motion is tilted from the  $c$ -axis by *ca.* 20° (Figure 3). This deviation occurs towards the OH group in Figure 3. Solvation around this OH group through weak hydrogen bonding of the type  $(CH_3)_2S=O \cdots HO-R$ , can be suggested as a possible reason for this deviation. The tilting angle  $\theta$  decreases with the temperature (Table 2). This is in agreement with the results expected, since the molecular interactions which cause solvation will be weakened on increasing the temperature.

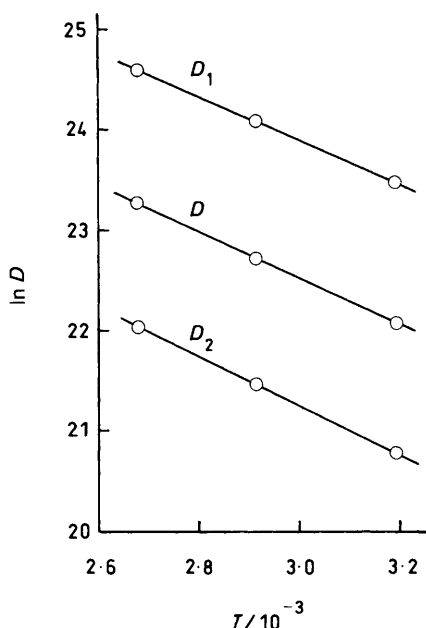
**Temperature Dependence of the Reorientational Rate Constants.**—The reorientational rate constants,  $D$ ,  $D_1$ , and  $D_2$ , increase with the temperature (Table 2). This tendency surely arises from the decrease in viscosity of the solvent. On the other hand, the ratio  $D_1/D_2$  is independent of the temperature within the estimated errors over the temperature range measured. The temperature dependence of the correlation time  $\tau_c$  is usually expressed by equation (1)<sup>8</sup> where  $E_a$  is the activation energy of

$$\tau_c = \tau_0 \exp(E_a/RT) \quad (1)$$

**Table 4.** Analysis of  $T_1$  data for the quaternary carbons of testosterone in  $[^2\text{H}_6]\text{DMSO}$  at 25 MHz.<sup>a</sup>

$\delta$ (ppm)	Site	Type	$N_\alpha^b$	$T_{1,\text{obs}}$			$T_{1,\text{calc}}^{\text{DD}}$			$T_1^{\text{oth}}$			$T_{1,\text{obs}}/T_{1,\text{calc}}^{\text{DD}}$		
				40 °C	70 °C	100 °C	40 °C	70 °C	100 °C	40 °C	70 °C	100 °C	40 °C	70 °C	100 °C
196.78	3	sp <sup>2</sup>	3	11.7	19.2	29.1	14.1	27.3	45.9	68.7	65.2	79.6	0.83	0.71	0.63
171.99	5	sp <sup>2</sup>	3	8.4	14.7	23.4	9.1	18.0	31.1	109.2	80.0	93.7	0.92	0.82	0.75
42.08	13	sp <sup>3</sup>	7	6.6	11.3	17.9	6.7	12.8	21.9	366.3	96.4	99.9	0.98	0.88	0.82
37.85	10	sp <sup>4</sup>	6	7.2	12.7	18.7	7.5	14.4	24.2	180.0	106.4	83.4	0.96	0.88	0.78

<sup>a</sup>  $T_1$ /s. <sup>b</sup>  $N_\alpha$  is the number of  $\alpha$ -protons.



**Figure 4.** Plots of  $\ln D$  vs.  $1/T$  which yield the activation parameters for the molecular reorientational motion for testosterone in  $[^2\text{H}_6]\text{DMSO}$ .

the reorientational motion. Equation (2) therefore holds for the rate constant  $D$ , since  $D$  is inversely related to  $\tau_c$ , i.e.,  $\tau_c = 1/6D$ .

$$D = D_0 \exp(-E_a/RT) \quad (2)$$

In these equations,  $\tau_0$  and  $D_0$  are the limiting values of  $\tau_c$  and  $D$ , respectively, which would be attained at very high temperatures. In fact, plots of  $\ln D$  vs.  $1/T$  show linear relations (Figure 4), and the activation energies are found to be 19.8, 18.2, and 21.8 kJ mol<sup>-1</sup> for  $D$ ,  $D_1$ , and  $D_2$ , respectively. These values are larger than those observed for the smaller molecules, e.g.,  $(\text{CH}_3)_2\text{SnCl}_2$  and its bipyridine complex in  $\text{CDCl}_3$ .<sup>9</sup>

**Rotation of the Two Methyl Groups.**—The barrier to internal rotation  $V_0$  can be estimated at each temperature, as described in the Results section, since the limiting rate constant  $D_{i0}$  is known for a methyl group. This value is ca. 9 kJ mol<sup>-1</sup> for the 18-methyl group and ca. 12 kJ mol<sup>-1</sup> for the 19-methyl group (Table 3). The rotation of the 19-methyl group is therefore hindered more than that of the 18-methyl group. This hindered rotation is also reflected in the lower values of  $D_i$  and shorter values of  $T_1$ . Quite the opposite result is observed for  $5\alpha$ -androstane (2):<sup>3</sup> here the 19-methyl group rotates faster than does the 18-methyl group. Such a discrepancy needs to be explained by a much more detailed study of methyl rotation in other similar compounds. The present results, however, may be in accordance with the proposed explanation:<sup>3</sup> that the

asymmetric placement of axial hydrogens may provide a rotameric energy well and thus slow down the internal rotation, since the OH proton located on the  $\text{H}_3\text{C}-\text{C}-\text{OH}$  framework can act as an axial hydrogen and thus enhance the symmetric placement as well as the rate of rotation of the 18-methyl group in testosterone.

**Relaxation of the Quaternary Carbons.**—In Table 1 the estimated values of  $T_1$  and  $T_{1,\text{calc}}^{\text{DD}}$  are no longer than those observed for the quaternary carbons, indicating a considerable contribution from sources other than dipole-dipole relaxation. If these contributions are denoted as  $T_1^{\text{oth}}$ , they can be estimated from equation (3). The fraction of the dipole-dipole relaxation

$$1/T_1^{\text{oth}} = 1/T_{1,\text{obs}} - 1/T_{1,\text{calc}}^{\text{DD}} \quad (3)$$

is defined as the ratio of the relaxation rate  $T_1^{-1}$ , i.e., it is expressed as  $(T_{1,\text{calc}}^{\text{DD}})^{-1}/(T_{1,\text{obs}})^{-1}$ , and hence as  $T_{1,\text{obs}}/T_{1,\text{calc}}^{\text{DD}}$ . These values are summarized in Table 4 for the quaternary carbons at each of three temperatures. Hitherto, the relaxation of quaternary carbons had been reported to be dominated by dipole-dipole relaxation, likely to be that of proton-bearing carbons.<sup>4</sup> This is the case at lower temperatures in the present case (measured at 25 MHz). On increasing the temperature, however, the contribution of the dipole-dipole relaxation decreases as seen from the ratio  $T_{1,\text{obs}}/T_{1,\text{calc}}^{\text{DD}}$  in Table 4. For quaternary carbons, relaxations *via* the chemical-shift anisotropy ( $T_1^{\text{CA}}$ ), the spin-rotation interaction ( $T_1^{\text{SR}}$ ), and the scalar coupling ( $T_1^{\text{SC}}$ ) are suggested as possible mechanisms other than the dipole-dipole relaxation.<sup>8</sup> Of these contributions,  $T_1^{\text{SC}}$  is significant for quaternary carbons attached to halogen<sup>8</sup> but is not discussed further here. The relaxation times  $T_1^{\text{CA}}$  and  $T_1^{\text{SR}}$  are expressed by equations (4) and (5),<sup>8</sup>

$$1/T_1^{\text{CA}} = (2/15)(2\pi\nu_0)^2(\Delta\sigma)^2\tau_c/\{1 + (2\pi\nu_0\tau_c)^2\} \quad (4)$$

$$1/T_1^{\text{SR}} = (2IkT/h^2)\{(C_{\parallel}^2 + 2C_{\perp}^2)/3\}\tau_c \quad (5)$$

where isotropic reorientational motion is assumed for the sake of simplicity. When an extreme narrowing condition  $2\pi\nu_0\tau_c \ll 1$  and a relation  $\tau_c\tau_j = I/6kT$  are considered, the above equations are modified to yield equations (6) and (7). An increase in

$$1/T_1^{\text{CA}} = (2/15)(2\pi\nu_0)^2(\Delta\sigma)^2\tau_c \quad (6)$$

$$1/T_1^{\text{SR}} = (\pi I^2/3h^2)\{(C_{\parallel}^2 + 2C_{\perp}^2)/3\}(1/\tau_c) \quad (7)$$

temperature will therefore result in an increase in  $T_1^{\text{CA}}$  and a decrease in  $T_1^{\text{SR}}$ , since  $\tau_c$  decreases with the temperature. It is seen from Table 4 that  $T_1^{\text{oth}}$  decreases with the temperature for the sp<sup>3</sup> carbons but is essentially independent of the temperature for the sp<sup>2</sup> carbons. This tendency can be interpreted, therefore, as indicative of the dominant contributions of  $T_1^{\text{SR}}$  in  $T_1^{\text{oth}}$  for the quaternary sp<sup>3</sup> carbons and the compensated

contributions of  $T_1^{\text{SR}}$  and  $T_1^{\text{CA}}$  for the quaternary  $\text{sp}^2$  carbons. Such an increased contribution of  $T_1^{\text{CA}}$  is reasonable for the  $\text{sp}^2$  carbons because of their large anisotropy in the chemical shift  $\Delta\sigma$  [equation (4)]. In this manner,  $T_1^{\text{CA}}$  and  $T_1^{\text{SR}}$  contribute significantly to  $T_1^{\text{oth}}$  and hence slightly to the observed  $T_1$  value for the quaternary carbons.

In conclusion we have shown, using testosterone as a model compound, that a computer-assisted method of analysis is useful for the interpretation of the relaxation data for carbon atoms, including quaternary carbon atoms. For example, information can be derived concerning the relaxation mechanisms of quaternary carbons, and activation energies can be obtained for the molecular anisotropic motion as well as for the methyl internal rotation.

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