9β,19-Cyclosterol Analysis by ^{1}H and ^{13}C NMR, Crystallographic Observations, and Molecular Mechanics Calculations

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Abstract: Using modern NMR techniques, including COSY, HOHAHA, 2D *J* spectrum, NOESY, HETCOR and HMBC, determination of ^{13}C T_1 spin−lattice relaxation times, and molecular modeling, permitted the full assignment of all ^{1}H and ^{13}C chemical shifts and geminal and vicinal coupling constants of cycloartenol. Stereochemical assignments related to the solution conformation of cycloartenol established unambiguously the nucleus assumes a *flat* structure and the side chain orients preferentially into a "right-handed" conformation, i.e., 20α-H atom is in front and opposing (in a 1,3-diaxial relationship with) the C_{18} angular methyl group thereby positioning C_{22} to the right of C_{20} (H_{20} trans oriented to H_{17}) in the usual view of the molecule. The crystal structures of two naturally occurring 9β,19-cyclopropylcycloartenol metabolites, 4-normethylcycloartenol and 4,4-dinormethylcycloartenol, have also been determined and found to possess a three-dimensional shape similar to that of cycloartenol. The biosynthetic implications of these findings are discussed in relation to the cyclization of squalene oxide to cycloartenol.

Introduction

An understanding of the preferred stereochemistry that gives rise to the physiological conformation of natural products is an essential prerequisite to studies on the biosynthesis and function of these molecules. The structural elucidation of complex natural products, particularly 9β,19-cyclophytosterols, has been unambiguously determined by a combination of spectral techniques including two-dimensional (2D) NMR, X-ray crystallography, and molecular modeling.[1] The formation of phytosterols involves the initial enantioselective cyclization of squalene oxide ((*S*)-2,3-epoxysqualene) to cycloartenol, followed by extensive modification of the tetracyclic monol to generate Δ^5-24-alkyl sterol end products.[2] The molecular details of the enzymic conversion of (*S*)-2,3-oxidosqualene to cycloartenol and the structure and conformation of the tetracyclic product resulting from cycloartenol synthase (2,3-oxidosqualene: cycloartenol-cyclase: EC 5.4.99.8) have been rationalized by several research groups[3] using stereochemical relationships of the parent cyclization reactions as envisaged by the Zurich school.[4] These observations were correlated with Dreiding models of 9β,19-cyclosterols, suggesting that cycloartenol and some related cycloartenol isomers (e.g., 10α-cucurbitadienol)[5] are "bent" structures. The putative bent 9β,19-cyclosterol structure was considered to be a unique phylogenetic trait related to the architectural suitability and sterol specificity of phytosterols which bind to enzymes that act on sterols.[6] Recent work on rationally designed inhibitors, enzyme purification and gene cloning of cycloartenol synthase,[7] and related sterol synthase enzymes has added to the interest in this area.[8]

The rationale for 9β,19-cyclosterols to be bent is based on the syn-cis configuration at the A/B and B/C ring junctions, resulting in an unfavorable interaction between the 9,10-bridge head and the 8β-hydrogen atom at C_8. Furthermore, on the basis of manipulation of ball-and-stick models, it was proposed that ring B in 9β,19-cyclosterols becomes a boat and the A/B/C rings orient in the chair−boat−chair conformation.[5] However, we examined the solution conformation of 30,31-dinorcycloartenol (24,25-dehydropollinstanol) and provided NMR evidence that suggested the preferred conformation of 9β,19-cyclosterols

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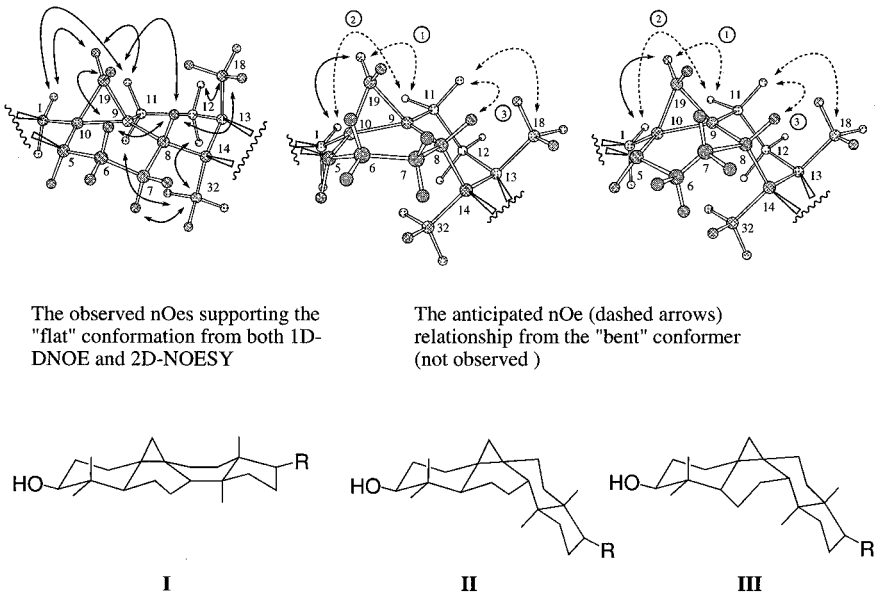

Figure 1. Flat conformer of cycloartenol with NOEs observed from DNOE and 2D phase-sensitive NOESY and anticipated NOE relationship for the bent conformers.

should be "flat" (pseudoplanar).[9] After our report appeared in the literature, Milon et al.[10] and Pascal et al.[11] reported that, on the basis of their interpretation of the NMR and molecular mechanics (MM) calculations on cycloartenol and specificity studies on sterol enzymes from corn, there was an equilibrium between two of three steroid-frame conformers of cycloartenol, e.g., I and II in Figure 1.

The three 9β,19-cyclosterol structures shown in Figure 1 are thought to represent the significant conformational variants generated by ring interconversion. I is similar to the crystallographically determined cycloartenol structure:[12] I, A/B/C rings are chair/half-chair/*twist-chair* conformer; II, A/B/C rings are the chair/half-chair/chair conformer; and III, A/B/C rings are the chair/boat/chair conformer. Of the three conformers, III was the bent conformer envisioned from the crude ball-and-stick models. The study by Milon et al.[10] agreed with our conclusions[9] that the 9β,19-cyclosterol in the conformation represented by III was not a viable structure. Somewhat surprisingly, II was calculated to be the lowest energy conformer; I and III were 1.5 and 4.9 kcal/mol higher in energy, respectively.[10] In none of the earlier NMR or MM studies on 9β,19-cyclosterols was the side chain of the sterol considered. Nonetheless, there has been much debate on the degree to which rotational isomerism about the 17(20)-bond of sterols and sterol-like molecules (e.g., euphol and tirucallol) may occur.[1c,5,13,14]

We now report for the first time excellent agreement between a set of crystallographically observed cycloartenol-type structures and their solution conformations deduced from the 2D-NMR analysis and MM/MD calculations. These experiments confirm and extend the observations of several groups[15] that show that the side chain is oriented to the "right" for phytosterols to serve as the physiologically active compound. In contradistinction to "right-handed" sterols, Corey et al.[16] recently demonstrated that the cyclization of squalene oxide to lanosterol generated a stable structure that is initially "left-handed". To account for this unusual behavior of structural isomerism in sterols, we propose that the cycloartenol synthase catalyzes ring annulation to form a flat structure that mimics the "left-handed" lanosterol. This structure undergoes further nuclear transformation to produce a 9β,10-cyclopropane ring, and rotational isomerism about the 17(20)-bond occurs to generate a "right-handed" structure of the type observed in solution and the solid state. Formation of the 9β,19-cyclopropane ring in the penultimate step in cycloartenol synthesis operates mechanistically, such that nucleophilic substitution at C-19 occurs with retention of configuration, thus allowing for lanosterol to be an enzyme-bound intermediate in the reaction pathway.

Results

Assignment of ^{13}C and ^{1}H NMR Chemical Shifts of Cycloartenol. Analysis of the one-dimensional (1D) 500-MHz spectrum of cycloartenol (with a free C-3 hydroxyl group) in $CDCl_3$ shows, in addition to H_{19} endo and H_{19} exo and the readily assigned methyl signals, only a few other signals that might be tentatively assigned (which is evident from Figure 2 showing a 2D NOESY of cycloartenol) due to extensive

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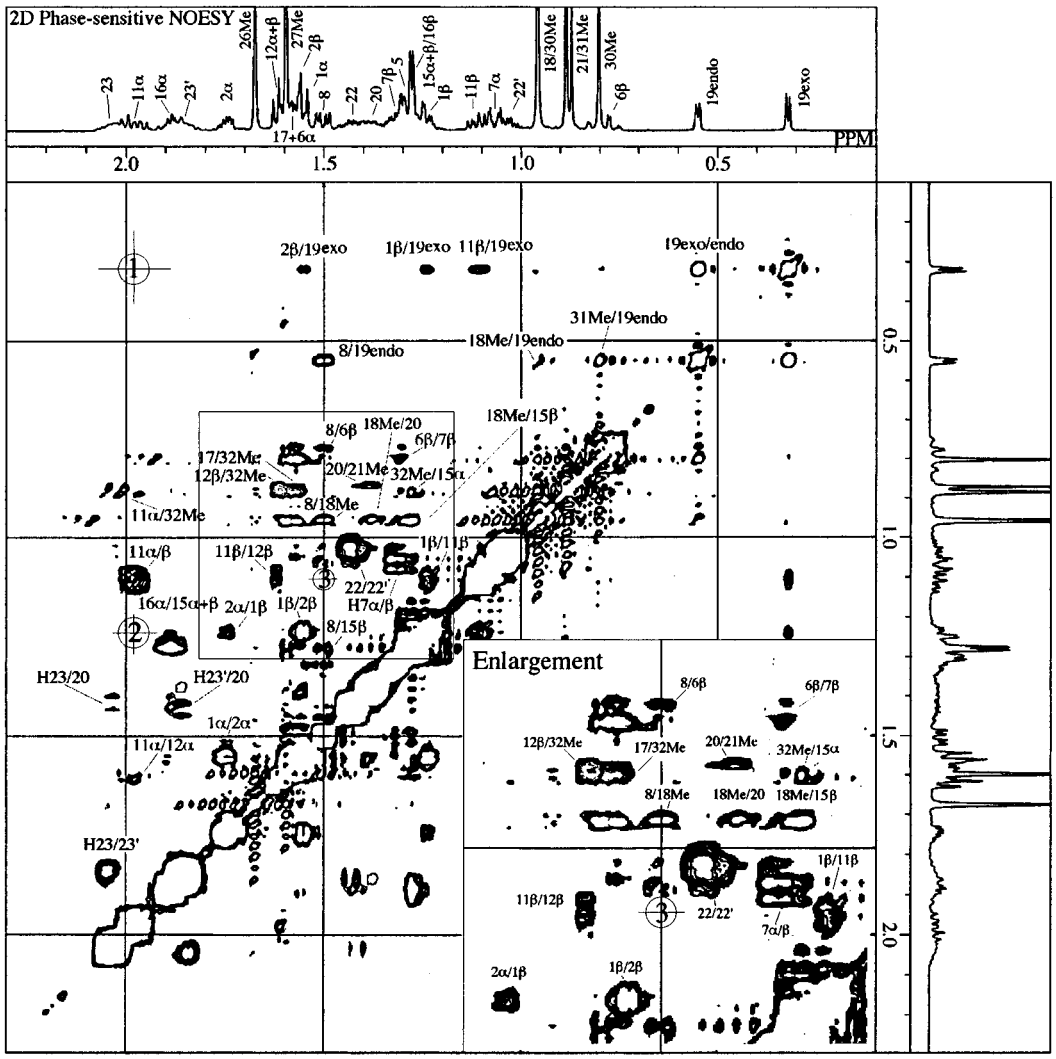

Figure 2. 2D phase-sensitive NOESY map of cycloartenol (the $\tau_{mix} = 600$ ms), recorded at 500 mHz in CDL_3. The anticipated NOE cross-peaks (nonexistence) for the bent in Figure 1 are marked by the numbers 1−3.

interproton couplings and severe overlappings resulting from the chemical shift similarity of the ring methylenes (the so-called methylene envelope operating between δ 1.0 and 2.0 ppm). The multiplicities of carbon signals were determined by the distortionless enhancement by polarization transfer (DEPT) method, as shown in Table 1. Because cycloartenol is viewed as both a sterol[3a] and a triterpene,[17] two different nomenclature systems are often used; as a result, the assignments on this molecule are somewhat confusing. We prefer to use the sterol numbering system which incorporates the *biosynthetic side chain rule* introduced by Popjak[20] and Nes,[3b] where the methyl group in squalene trans to C_{23} which is derived from C_2 of mevalonic acid becomes C_{26} in the sterol, not the *cis*-isopropylidene carbon as some investigators recognize.[17] The methyl groups in the nucleus are recognized as C_{30} (C4α, equatorial), C_{31} (C4β, axial), and C_{32} (C14β, axial) according to the classic sterol nomenclature system rather than as C_{28}, C_{29}, and C_{30}, which has been adopted by IUPAC.[3b,d] To resolve the ambiguous structural assignments and to assign all the proton signals of cycloartenol, several different proton mapping techniques (COSY, HOHAHA coupled with 2D *J* spectra) were used.[21] On the basis of the assigned proton signals, an HETCOR experiment then gave out the corresponding carbon assignment. For cycloartenol, four substructures were established from COSY, HOHAHA, and HETCOR experiments: $C_1-C_2-C_3$, $C_5-C_6-C_7-C_8$, $C_{11}-C_{12}$, and C_{15}, C_{16}, C_{17}, C_{20}, (C_{21}), C_{22}, C_{23}, C_{24}. After the assignments of the protons and protonated carbons were established, the quaternary carbons were found by means of the ^{1}H-detected heteronuclear multiple-bond correlation (HMBC).[21] The spatial orientation of the protons was determined from difference NOE spectroscopy (DNOE) and 2D phase-sensitive NOESY.

Relative Stereochemistry and Solution Conformation of the Cycloartenol Nucleus. After the assignment of the protons and carbons, several approaches were used to probe the three-dimensional (3D) structure of cycloartenol molecule in solution: (i) NOE experiments: difference NOE spectroscopy

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Table 1. ^{13}C and ^{1}H Chemical Shifts and ^{13}C Spin−Lattice Relaxation Times (T_1) for Cycloartenol (125 MHz for ^{13}C or 500 MHz for ^{1}H in $CDCl_3$ at 303 K)[a]

carbon	^{13}C	DEPT	^{1}H	av T_1	NT_1
1	32.00	CH_2	1.25 β, 1.55 α	0.93	1.86
2	30.42	CH_2	1.57 β, 1.75 α	0.78	1.56
3	78.87	CH	3.28 α	1.76	1.76
4	40.51	C		11.46	11.46
5	47.15	CH	1.29 dd (12.7, 4.4)	1.76	1.76
6	21.14	CH2	0.79 β, 1.59 α	0.88	1.76
7	26.03	CH_2	1.08 α, 1.32 β	0.90	1.80
8	47.99	CH	1.51 dd (12.5, 4.9)	1.51	1.51
9	20.05	C		11.15	11.15
10	26.12	C		11.55	11.55
11	26.53	CH_2	1.11 β, 1.99 α	0.93	1.88
12	32.94	CH_2	1.62 α + β	0.94	1.86
13	45.32	C		11.09	11.09
14	48.83	C		13.27	13.27
15	35.60	CH_2	1.28 α + β	0.86	1.72
16	28.16	CH_2	1.28 β, 1.90 α	0.88	1.76
17	52.32	CH	1.59 α	1.62	1.62
18	18.03	CH_3	0.97	0.83	2.49
19	29.91	CH_2	0.33 d (exo, 4.2) 0.56 d (endo, 4.2)	0.90	1.80
20	35.91	CH	1.38	1.51	1.51
21	18.26	CH_3	0.89 d (7.0)	1.42	4.26
22	36.38	CH_2	1.05, 1.44	0.90	1.80
23	24.97	CH_2	1.86, 2.04	1.20	2.40
24	125.29	CH	5.10 m	2.46	2.67
25	130.89	C		13.47	13.47
26	17.64	CH_3	1.68 d (1.0)	8.26	24.78
27	25.72	CH_3	1.61 s	3.17	9.51
30	25.46	CH_3	0.97 s	1.03	3.09
31	14.02	CH_3	0.81 s	1.35	4.05
32	19.33	CH_3	0.89 s	3.81	11.43

[a] 1. Proton assignments based on ^{1}H NMR, DQF−COSY, 2D-HOHAHA, 1D DNOE, and 2D phase-sensitive NOESY. 2. The proton orientations were determined from 1D NOE difference spectra and 2D phase-sensitive NOESY. 3. ^{13}C assignments were based on DFQ-COSY, 2D-HOHAHA, HETCOR, and HMBC.

(DNOE) and 2D NOESY experiments were performed to establish the spatial orientation and interproton distances of protons on each face of the nucleus,[22a] followed by an analysis of the geminal proton chemical shifts and vicinal proton couplings. Axial protons are well-known to resonate at a higher field than equatorial protons in the cyclohexanes, except in the vicinity of substituents. The same is generally true for protons on the steroid ring system.[22b] The Karplus dihedral angle relationship has been the strongest tool in conformational analysis by proton NMR.[22c] Local conformation can be read out by analyzing the geminal and vicinal proton couplings nearby. Chemical shift assignments and coupling constant determinations were established using 2D *J* spectra related to the COSY and HOHAHA experiments. It was necessary to use computer simulation to confirm and refine the chemical shifts and the coupling constants read from the 2D *J* spectrum to establish and confirm chemical shifts and their corresponding coupling patterns. Knowledge gained regarding the spin−spin coupling of the stereochemistry of nearby protons confirmed the conformational analysis deduced from NOE studies. (ii) HMBC experiments: as with the proton−proton vicinal coupling constant, numerous theoretical and empirical studies

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Table 2. Connectivities Established by 2D Phase-Sensitive NOESY

proton	NOE obsd
H-3	H-1α, H-2α, H-5, 30-Me
31-Me	H-2β, H-19 (endo)
H-6β	H-7β, H-8, H-19 (endo)
H-19 (endo)	H-6β, H-8, 18-Me, H-19 (exo), 31-Me
H-19 (exo)	H-1β, H-2β, H-11β, H-19 (endo)
18-Me	H-8, H-12β, H-15β, H-20 H-19 (endo), H-19 (exo)
30-Me	H-5, H-6α, 31-Me
32-Me	H-7α, H-11α, H-12α, H-15α, H-17
21-Me	H-17, H-20, H-23
H-24	H-22, H-23, H-23′, 26-Me
26-Me	H-23, H-24

suggest that similar behavior may exist for three-bond carbon−proton couplings and usually a maxima of $^{3}J_{CH}$ was obtained at 0° and 180° and a minimum near 90°.[22d] Three-bond long-range proton−carbon coupling has been successfully applied to the conformational analysis of sterol ring system.[22e] Thus, stronger cross-peaks can be observed in HMBC experiments when a H−C−C−C fragment possesses a dihedral angle close to 180°, results which depend on conformational preferences in the cyclosteroid molecule.

The three distinct conformations that generate the bent and flat cycloartenol structures were distinguished from one another initially by DNOE and 2D NOESY experiments (Table 2). The NOE network that can be expected from the different cycloartenol conformers are shown in Figure 1. The DNOE and 2D NOESY experiments eliminated structures II and III (Figure 1) as possible dominant conformers of cycloartenol in solution, suggesting that cycloartenol exists as the flat structure (I in Figure 1) in solution. The key features on cycloartenol that indicate the structure assumes a flat conformation are the protons associated with C_1, C_3, C_6, C_8, C_{11}, C_{12}, and C_{19} in rings A/B/C and the angular methyl groups C_{18} and C_{32} in ring D.

A Ring. H_3 at δ 3.22 is known to be an axial proton on the α-face (back) of the nucleus. Irradiation of H_3 induced NOEs at δ 1.75 (2.4%, $H_{2\alpha}$), 1.55 (8%, $H_{1\alpha}$), 1.30 (12.3%, $H_{5\alpha}$), and 0.97 (30-CH_3, equatorial). Therefore, all of these protons are oriented to the α-face of the molecule. The strong positive NOEs of the $H_{1\alpha}$ and H_5 indicated a 1,3-diaxial relationship between H_3 and $H_{1\alpha}$ and H_3 and H_5. Irradiation of C-31 methyl group at δ 0.81 gave NOEs at δ 1.57 ($H_{2\beta}$) and 0.56 (H_{19endo}). Due to the coincidence of the $H_{6\beta}$ and H_{31CH3} signals, we failed to observe the spatial relationship between $H_{6\beta}$ and H_{31CH3}. The NOEs induced at δ 1.32 ($H_{7\beta}$) and 1.51 (H_8) were found from the coirradiation of the $H_{6\beta}$. The above evidence shows that the A ring should adopt a *chair* form.

B Ring. Irradiation of the H_{19endo} showed NOEs at $H_{6\beta}$ (δ 0.78), H_8 (δ 1.51), H_{31CH3} (δ 0.81), and H_{18CH3} (δ 0.97). Additionally, the coupling constants $J_{5,6\beta}$, $J_{6\beta,7\alpha}$, and $J_{7\alpha,8}$ were all about 12.0 Hz, indicating the axial orientation of these protons. From the NOE experiments, we found that the axial hydrogen atom at C_6 (β-oriented and above the plane of the molecule) was shielded to a significant extent, due to influence of the 9β,19-cyclopropane ring projecting from the β-face (front in the usual view of the molecule) whereas the axial proton on C_7 is not strongly influenced by the 9β,19-cyclopropane ring. These results clearly show that structure III was disfavored, confirming our earlier NMR studies on 30,31-dinorcycloartenol.[9] Our more extensive NOE study revealed that the H_{19exo}, $H_{1\beta}$ (equatorial), and $H_{11\beta}$ (quasi-equatorial) are positioned in a triangular arrangement and within the NOE distances. The $H_{1\beta}$ resonated at a higher field than $H_{1\alpha}$ consistent with a well-

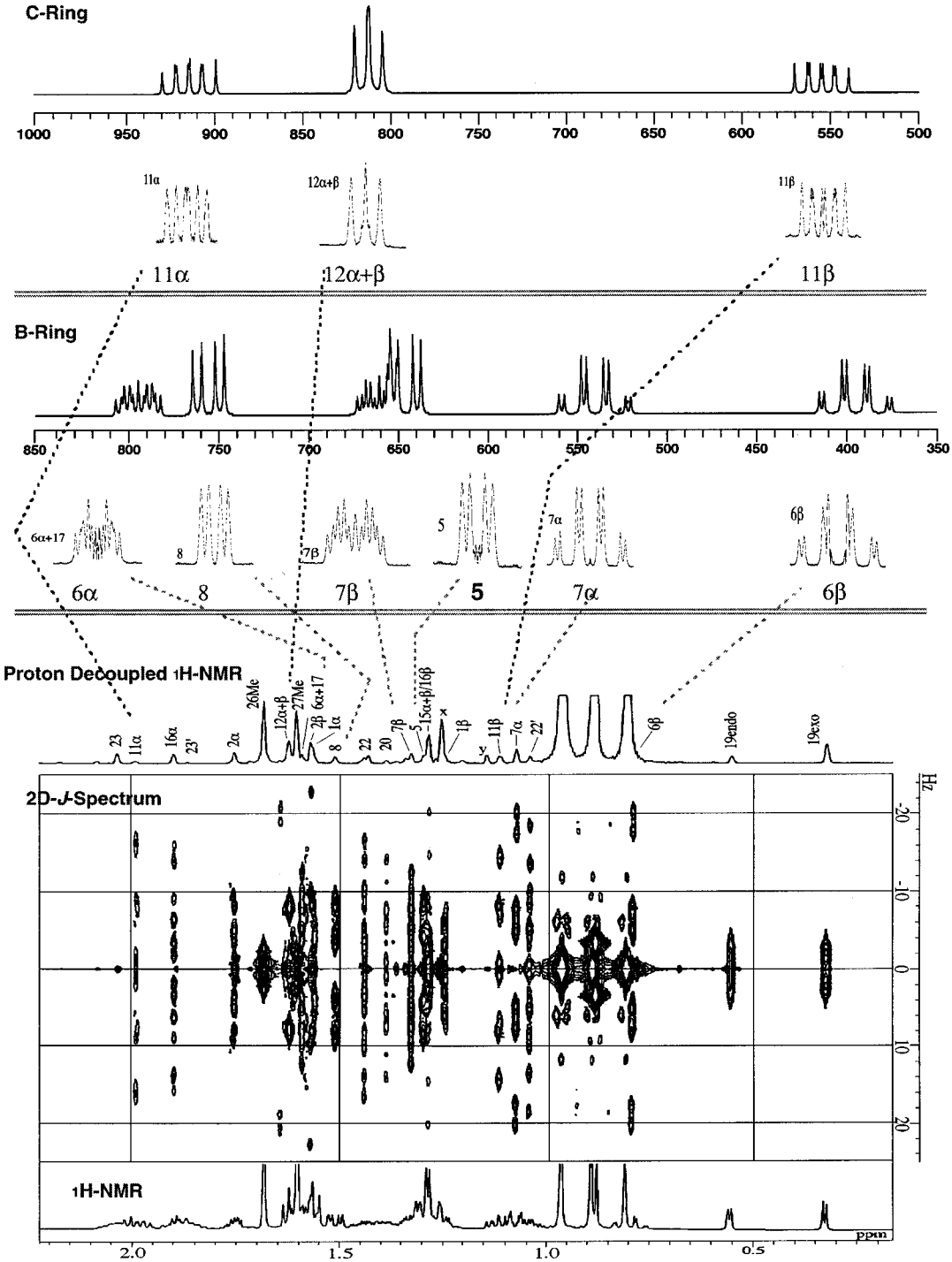

Figure 3. 2D *J* spectrum with *J* spectra of protons from B and C rings, along with "proton-decoupled" spectrum (x and y mark artifacts) and the computer-simulated spectra.

defined chair A ring structure, suggesting that the $H_{1\beta}$ undergoes a shielding effect from the 9β,19-cyclopropane ring. The same observation was found for $H_{11\beta}$, which resonated at δ 1.11 ppm. Thus, the orientation of the B ring may be assumed to be *half-chair*.

C Ring. Assuming the C ring orients into the chair form as suggested by Milon et al.,[10] then the $H_{11\alpha}$ should be equatorial and lie near the axis of the diamagnetic anistropy perpendicular to the plane of the 9,10,19-three-membered ring (II in Figure 1). In the C ring conformation, the $H_{11\alpha}$ may move to high field, but this proton was found to resonate at low field at δ 1.99 and the $H_{11\beta}$ resonated at relatively high field at δ 1.11, due to the shielding influence from the 9β,19-cyclopropane ring. Milon et al.[10] claimed that a clear distinction between I and II (Figure 1) may come from an analysis of the spin system of $H-C_{11}/H-C_{12}$. Unfortunately, as Milon et al.[10] recognized and observed here, the two H_{12} protons appear to be magnetically equivalent and give a triple splitting pattern which thereby complicates matters. We circumvented this problem by analyzing the HMBC spectrum of cycloartenol, which shows $H_{11\beta}$ and C_8 and $H_{11\beta}$ and C_{13} give a dihedral angle about 180° (strong cross-peaks in the HMBC, Figure 4) whereas there is no or weak correlation between $H_{11\alpha}$ and C_8 and $H_{11\alpha}$ and C_{13} (corresponding to about 90°). Irradiation of the H_{19exo} induced NOEs at $H_{1\beta}$ (δ 1.25) and $H_{11\beta}$. Irradiation of 18-CH_3 (δ 0.97) gave NOEs at H_{19} (endo and exo), H_8, and H_{20} (δ 1.38). Irradiation

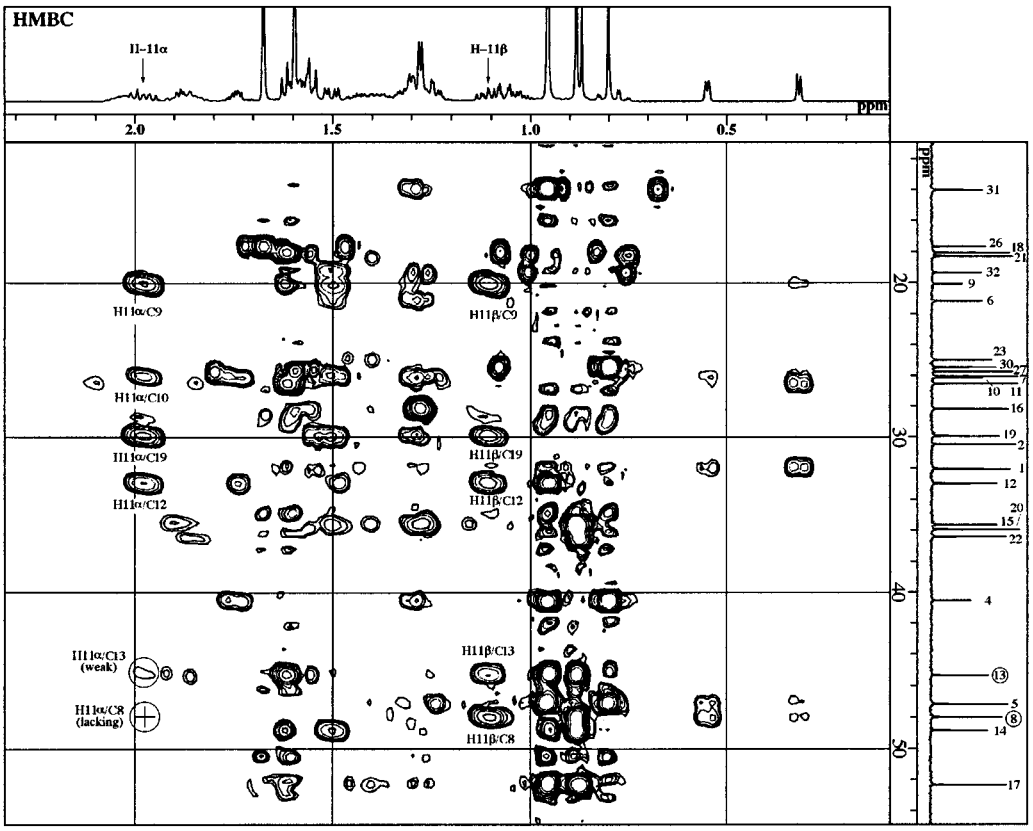

Figure 4. HMBC spectrum of cycloartenol showing long-range (two or three bond) couplings. Selected peaks discussed in text are marked.

Table 3. Germinal and Vicinal Coupling Constants in Rings B and C (in Hz)[a]

		^3J		
proton	^2J	ax/ax	ax/eq	eq/eq
5/6α			4.4	
5/6β		12.7		
6α/6β	|12.5|			
6α/7α			2.9	
6α/7β				4.9
6β/7α		12.4		
6β/7β			2.6	
7α/7β	|12.4|			
7α/8		12.5		
7β/8			4.9	

proton	^2J and ^3J	proton	^2J and ^3J
11α/11β	|14.8|	11β/12α	7.4
11α/12α	8.2	11β/12β	8.6
11α/12β	7.4	12α/12β	0

[a] Couplings read from 1D ^{1}H NMR and 2D *J* spectrum.

of 32-CH_3 on the α-face gave NOE at H11α (1.98 ppm). The abnormal behavior of the chemical shifts and coupling patterns (Table 3) from the four spin systems of H_{11} and H_{12} combined with the structural information from NOE and HMBC suggest that the solution conformation of the C ring of cycloartenol is a *twist-chair*.

D Ring. The axial and equatorial distinction is blurred in the five-membered and conformationally mobile ring D, thus interfering with an exacting analysis of its conformation from NOE study.[25] Nonetheless, as shown in Table 2, strong NOEs were observed between H_{32} and $H_{11\alpha}$ and $H_{12\alpha}$ and between H_{18} and $H_{12\beta}$, suggesting that the D ring was in a pseudoplanar conformation.

To establish further whether there was interconversion of the cycloartenol structure from a flat into a bent shape, a C-13 spin−lattice relaxation time (T_1) study was performed on cycloartenol. Spin−lattice relaxation time studies on steroids that are structurally related to the title compounds have shown that the side chain and methyl carbons undergo internal rotational motions in addition to the overall tumbling motions of the sterol molecule.[26] The motional characteristics of the backbone carbon atoms of these molecules are nearly isotropic as indicated by their NT_1 values. On the basis of the relaxation times for cycloartenol (Table 1), the averaged NT_1 value for the molecule is 1.74 s. All of the ring CH and CH_2 carbon atoms exhibit values with experimental error of the average value. That is, the T_1 values of the protonated ring backbone of cycloartenol are inversely proportional to the number of attached hydrogens. Therefore, the rotational motion of the ring backbone is essentially isotropic. The carbon atoms of the ring backbone with one attached proton have T_1 values in the range 1.51−1.76 s, whereas the carbon atoms with two direct C−H interactions have T_1 values ranging from 0.86 to 0.94 s. All of the methyl carbon atoms show direct evidence of internal rotation with the NT_1 times much larger than the averaged NT_1 values of the ring carbon atoms. The NT_1 value of C_{20} (1.51 s) indicated that this carbon undergoes no internal motion; therefore, C_{21} should be comparable with those of the ring methyl carbon atoms. Among these methyl carbon atoms, C_{18} is the fastest relaxing carbon atom with a T_1 of 0.83 s (NT_1, 2.49): this methyl suffers strong interaction with the methylene group of C_{19} and a methyl group at C_{21} as shown from DNOE and 2D NOESY as well the four/three diaxial neighbor proton

(23) Braunscheweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521.
(24) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093.
(25) Griffin, J. F.; Duax, W. L.; Weeks, C. M. *Atlas of Steroid Sturcture*; Plenum Press: New York, 1984.
(26) Allerhand, A.; Doddrell D.; Komoroski, R. *J. Chem. Phys.* **1971**, *55*, 189.

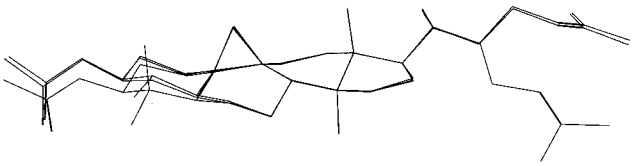

Figure 5. Superposition of a least-squares fit (19 steroid nucleus atoms of the crystallographically determined structures of the 3-OH acetates of cycloartenol,[12] 31-norcycloartenol, and 30,31-dinorcycloartenol (this report). The three compounds differ in substitution of methyl groups on C4, two, one, or none, respectively. When two methyl groups are on C4, the A ring is slightly flattened. Note, however, that the B, C, and D ring conformations are identical in the three structures.

interactions. These interactions probably provide a higher barrier to internal rotation, thus reducing the spin-rotation contribution. The next fastest relaxing carbon atoms are the geminal 4,4-dimethyl C_{30} (T_1, 1.09 s; NT_1, 3.09 s) and C_{31} (T_1, 1.35 s; NT_1, 4.05 s) groups. The longest T_1 value is observed for C_{32} (T_1, 3.81 s: NT_1, 11.43). This is probably due to the absence of nearest neighbors and the pseudoplanar conformation causing the carbons to relax slowly.

Relative Stereochemistry and Solution Conformation of the Side Chain of Cycloartenol. The location of the chemical shift (δ 0.89, doublet) for H_{21} in the 1D NMR spectrum (Table 1) suggested the 20*R* stereochemistry and the "right-handed" side chain.[13] The solution conformation of the side chain was confirmed from the dihedral angles between vicinal protons as deduced from the coupling constants obtained via the Karplus relationships. As necessary this analysis was supplemented by internuclear distances determined from NOE data.[1b] The preferred conformation of the side chain was determined by difference NOE and 2D NOESY experiments which indicated H_{18} and H_{20} were spatially related (Table 2). Irradiation of the C_{21} methyl group generated an NOE at both C_{23} protons, suggesting that the methyl group and pair of H_{23} protons are on the same side of the molecule. Irradiation at H_{24} gave a strong NOE at the methyl group at δ 1.68 assigned to C_{26}. 2D NOESY gave strong NOEs between H_{24} and $26CH_3$ and H_{24} and H_{22}. Rather weak NOEs were observed between H_{24} and H_{22} (*pro-S hydrogen atom*). These NOE relationships indicated that the olefinic proton at C_{24} is on the same side of the side chain with the two protons on C_{22} and they are in the space near one of the protons. The above NOE relationships indicate that the side chain orients preferentially into one or both (i.e., equilibrate) zigzag systems shown in the crystal structures of 9β,19-cyclosterols (Figure 5). We refer to these two principal side chain conformers as staggered (C_{22} trans oriented to C16) and pseudocyclic (C_{22} cis oriented to C16).[6b] We have shown that the side chain conformation bound by the sterol methyl transferase influences the stereochemistry of the reaction course.[27] The coupling constant of 10.5 Hz between H_{17} and H_{20} (Table 4) proved that an antiperiplanar relationship exists between these hydrogen atoms, thus giving the "right-handed" orientation (Figure 6). The T_1 values of the side chain increase along the side chain (Table 1), suggesting increased internal motion in the side chain as the terminal isopropyl group is approached. A similar conclusion regarding the rotational state of the side chain (C_{22} to $C_{26/27}$) has been deduced from crystallographic studies on sterols.[1a,16b] The summation of these results suggests that there is a significant degree of rotational isomerism occurring about the terminal C−C bonds in the sterol side chain, whereas there is restricted rotation in the proximal segment about the 17(20)-bond.

(27) (a) Zhou, W.; Guo, D.; Nes, W. D. *Tetrahedron Lett.* **1996**, *37*, 1339. (b) Guo, D.; Jia, Z.; Nes, W. D. *J. Am. Chem. Soc.* **1996**, *118*, 850. These references as well as refs 15a,b have shown that the sterol side chain structure bound to the yeast, sunflower and corn sterol methyl transferase enzymes approximate the pseudocyclic conformation.

Table 4. Selected Coupling Constant for the Side Chain[a]

proton	17	21	22	22′	23	23′
20	10.5	7.0	9.0	2.8		
22				|14.0|	10.1	4.8
22′					6.5	10.1

[a] Couplings read from 1D ^{1}H NMR and 2D *J* spectrum.

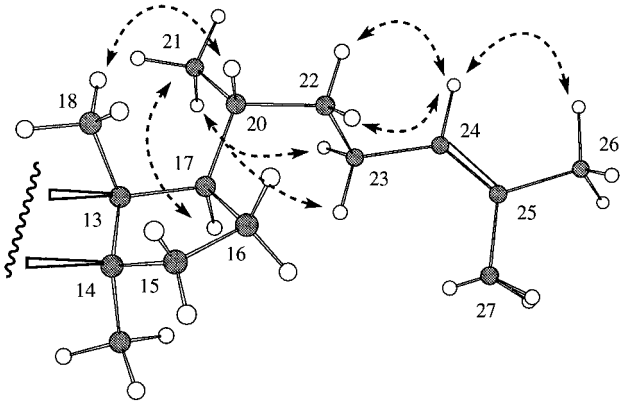

Figure 6. NOE relationship observed from DNOE and 2D NOESY studies.

Crystallographic Studies. Since the possibility was raised[10,11] that the degree of substitution at C_4 may influence conformational isomerism in the nucleus, we isolated 31-norcycloartenol and 30,31-dinorcycloartenol from cactus pollen and obtained their X-ray crytallographic structures.[28] As shown in Figure 5, the crystal structures of the acetates of the C-4 mononor and dinor analogues of cycloartenol acetate are compared with the previously determined X-ray crystallographic structure of the parent compound.[21] The three compounds possess a 14-methyl group, which, as will be shown later, may contribute to the conformation of 9β,19-cyclosterols (see Discussion). Two differences are seen between the three cycloartenol acetate structures with C_4 (2H) (*A*), C_4 (CH_3) (*B*), and C_4 ($2CH_3$) (*C*). That is, compared to the other two structures, when there are two methyl groups attached to C_4, a slight flattening of the A ring occurs and the tilt of the 17(20)-bond occurs directing the conformation of side chain into a pseudocyclic conformation. In the two structures reported here and in cycloartenol itself, the 8β hydrogen was found to make normal contacts of 2.30, 2.32, and 2.32 Å with the closest hydrogen atom on C_{19}. Apparently, the half-chair conformation accommodates this interaction. The ring conformations observed from these X-ray structures agree with the solution properties of cycloartenol and 30,31-dinorcycloartenol[10] showing a flat structure.

The side chain is described by the torsion angles defined in Table 4.[21] Structures *B* and *C* shown in Figure 5 are observed in an all-trans conformation, whereas *A* incorporates gauche torsion angles, suggesting that the side chain assumes a pseudocyclic conformation. The conformational flexibility of the side chain in the two structures is similar to that found in crystal structures of cholesterol and its derivatives.[1a] In all of these structures severe steric congestion between the C_{18} methyl group and the side chain attached at C_{17} has contributed to restricted rotation of the 17(20)-bond of sterols.

Application of Force Field Calculations to Cycloartenol Conformation. Molecular dynamic (MD) and molecular

(28) Griffin, J. F.; Nes, W. D.; Allinger, N. L. *INFORM* **1994**, (A) *5*, 509.

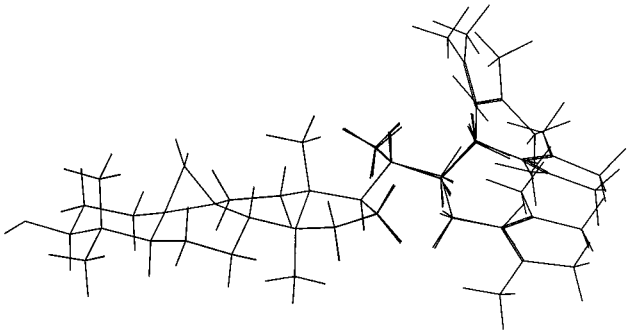

Figure 7. Overlay of the six most stable conformers (within 1.00 kcal/mol of the global minimum) from MCSD conformation search.

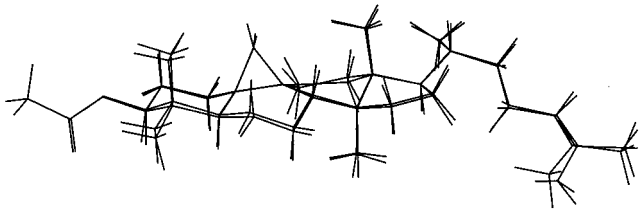

Figure 8. Superimposition of one of the most stable conformers from molecualr simulation with the crytallographically determined structure of cycloartenol acetate.[12] rms is 0.013 Å.

mechanics (MM) simulations can be used to obtain additional insight into conformational processes that have been detected by NMR. In an earlier study in cooperation with Prof. N. L. Allinger,[28] we used MM3 to study the conformation of cycloartenol. However, for those studies, the side chain was given a fixed (experimental) conformation and was not considered significantly in the calculations. The MM3 calculations show that the crystallographically observed structure is the most stable as an isolated molecule. The MM3 calculations predict that there is a second stable bent conformer 2.2 kcal/mol higher in energy resulting in a 9:1 ratio of the two at room temperature. Unfortunately, these predictions failed to indicate which (or both) of the bent conformers was present at room temperature.

In the present study, our calculations were performed with MacroModel software package (version 5.0) Conformation searches were performed by mixed-mode Monto Carlo/stochastic dynamics (MC/SD) simulations. High-temperature simulation was carried out at 2000 and 900 K with cycloartenol starting from either the flat or bent conformations. In both cases the MC/SD simulation indicated that cycloartenol gave the same global minima, generating a flat conformation. This conformation was close to the crystal structure which is the minimum energy form. Simulation at 2000 K found the most stable conformers four times among the 100 conformers sampled from MC/SD simulation, whereas simulation at 900 K gave the most stable conformer eight times. Of special interest was that when the most stable conformer from high-temperature simulation is further subjected to MC/SD at 298 K, the simulation gave out the same conformers 20 times out of the 100 conformers sampled. There are six conformers within 1.00 kcal/mol (4.18 kJ/mol) of the global minima. All of the six conformers assume similar flat geometries, and the side chains were found to assume similar "right-handed" side chain conformations, but differed in their side chain orientations with respect to rotational isomerisms about C_{22} and C_{23}. (Figure 7). At least one of these structures possess the crystallographically determined structure (rms is 0.013 Å; note superimpositions shown in Figure 8) and which we propose is the structure bound to sterol methyl

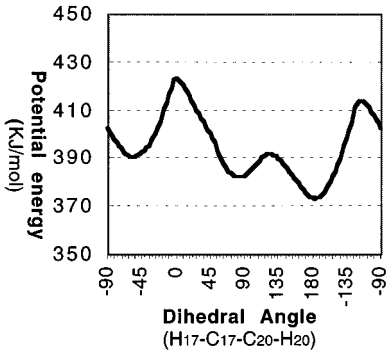

Figure 9. Dependence of the potential energy (at 0 K) on torsional angle $H_{17}-C_{17}-C_{20}-H_{20}$ of cycloartenol.

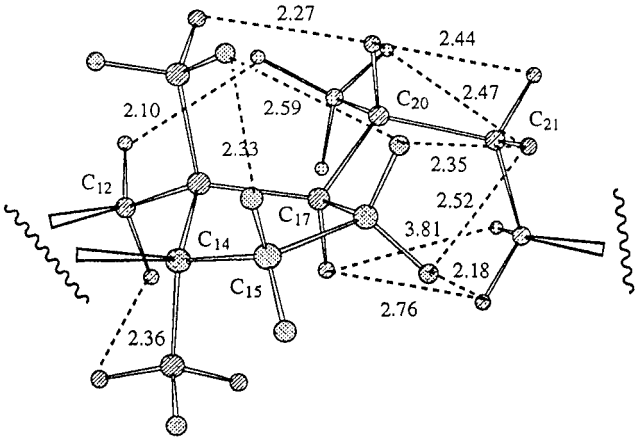

Figure 10. Partial structure of cycloartenol in its lowest energy rotamer around the $C_{17}-C_{20}$ bond. The dihedral angle $H_{17}-C_{17}-C_{20}-H_{20}$ is −176.8°. Calculated nonbonded distances were given in angstroms.

transferase enzymes;[15b] none were in the all-staggered orientation that would be expected for optimal fit of the sterol in the membrane.

The side chain orientation was investigated further with the input starting structure assigned from the most stable global minimum from the above MC/SD simulation. The $C_{17}-C_{20}$ bond was rotated through 360° at a 3° interval and energy minimized. A steric profile at 0 K relative to the dihedral angle $H_{17}-C_{17}-C_{20}-H_{20}$ is plotted in Figure 9. Inspection of the curve revealed that the different orientations of the side chain had significant influence on the steric energy of the molecule. The lowest energy minimum corresponding to a $H_{17}-C_{17}-C_{20}-H_{20}$ dihedral angle of −178.6° results in C_{21} and C_{22} groups to orient pseudo-equatorially on the back side, generating a "right-handed" side chain (Figure 10). A theoretical coupling constant calculated by the Altona equation gives a *J* value of 11.8 Hz, which is similar to the experimentally determined 10.5 Hz from NMR. Jiang et al. have recently shown, based on the coupling constant between H_{17} and H_{20} (10 Hz) and an NOE experiment between H_{18} and H_{20}, that the configuration at C_{20} is *S*-oriented and the preferred conformation of euphane-type steroids is one that possesses a left-handed structure in solution.[13d] Thus, for right-handed sterols, the anti-$H_{17}-H_{20}$ conformer seen in both the solid state and solution may be considered the ground-state conformation. Osawa et al. studying the steric energies that exist between "left-handed" and "right-handed" sterols (either as the *20S- or 20R*-compounds) determined that the former conformer is about 12 kcal/mol higher in steric energy than the latter structure,[1c] hence the natural proclivity to assume the "right-handed" structure.

Discussion

There is a clear association between the structure of 9β,19-cyclosterols and their biological properties, which are somewhat different from lanosterol and sitosterol.[6a,15a−d] The solid-state database of 16 9β,19-cyclosteroids in the Cambridge Crystallographic Database all show essentially the same nuclear conformation in the B and C rings, a 6β,7α-half-chair B-ring conformation and an 13β,14α-half-chair (twist-boat) C ring forming a flat molecule similar to lanosterol and sitosterol.[21,28,30] The putative arc-shaped conformation (bent) which changes the spatial disposition of the 14-methyl group and would account for the observed differences in NMR and activity does not exist. We are led to the conclusion that the minimum energy conformation of the cycloartenol frame should be a flat molecule with the B and C rings as in the crystal structures, which indeed is what our MM/MD simulations indicated.

Most interesting is that, when the molecular structure of lanosterol is compared to that of cycloartenol and sitosterol, we observe that lanosterol is the more bent and crowded molecule.[1a,15b] Minor differences in conformational transmission effects in these structures affect the tilt of the 17(20)-bond and hence the side chain orientation and affects also the tilt of the 3β-hydroxyl group; essential features which we have shown regulate sterol specificities.[15] The importance of the 4-methyl group is clear (structures *A*, *B*, and *C* in Figure 5), it affects the hydrogen bonding abilities of the C-3 hydroxyl group. However the importance of the 14-methyl group is problematic. Since the bent 9β,19-cycloartenol structure does not exist, the 14-methyl group orientation cannot be the major cause of the difference in biological properties between cycloartenol and lanosterol.[6c,15] Nonetheless, we found that the 14-methyl group may be important to the conformation of 9β,19-cyclosterols. When cycloartenol was incubated with the sterol auxotroph (yeast) GL7, it was converted to ergosterol and to a novel Δ^7-9β,19-cyclosterol that lacked a 14-methyl group.[31] From the chromatographic and spectral properties on the new 9β,19-cyclosterol, we concluded that the compound was bent. Related Δ^7-9β,19-cyclosterols that retain the 14-methyl group have been shown to be flat in solution and the solid state.[32] To account for the fact that some 9β,19-cyclosterols may assume a bent conformation whereas others may assume a flat structure, we propose that the methylene bridge on C_9-C_{10} imposes steric strain on the B and C rings, such that the C ring must adopt a conformation of least hindrance. The entropic balance between a 14-methyl group which tends to balance the repulsion from the bulky methyl groups on either face at C_{14} and at C_{13} and the surrounding hydrogens that fit between the 4β-methyl and C_{18} methyl groups determine the conformational status of the molecule. The structural elements axial to the nucleus coupled with the internal forces from the C ring are expected to promote chair formation or an equivalent low-energy structure (half-chair; twist-boat).

In conclusion, we comment on the cyclization of squalene oxide to cycloartenol in view of new data and insights from the studies on the cyclization of squalene oxide to lanosterol by Corey and co-workers.[8] The overall stereochemistry of the cyclization of 3(*S*)-epoxy squalene to the asymmetrical 9β,19-cyclotetracyclic product with a A/B trans ring junction and a 3β-hydroxyl group was unambiguously established by determining the enatioselectivity for 3(*R*)- and 3(*S*)-epoxysqualene as alternative substrates for the squalene oxide-to-cycloartenol synthase.[2a] However, given the relative stereochemical elements of the folding pattern (asymmetrical induced coil), presumed concerted nature of the cyclization process, and 1,2-rearrangements of carbocations occurring only with antiperiplanar alignments, the mechanism that generates the C-20 stereochemistry and stereochemisry of the 9β,19-cyclopropane ring of cycloartenol continues to be problematic. Two possible routes to generate cycloartenol from squalene oxide may be considered: (i) Covalent attachment of protosteroid intermediates with the synthase, the so-called "X^--group mechanism" proposed by Cornforth and others.[33] This mechanism requires that the substrate assume a conformation bound to the synthase that approximates the structure of *trans-syn-trans-anti-trans-anti*-1,2-cyclopentanoperhydrophenanthrene, which generates an intermediate with a 17α-side chain and a prochiral 20*S*-stereochemistry. Cyclization proceeds nonstop resulting in the formation of a C-20 protosteroid cation intermediate that is *covalently* attached to the synthase (rotation about C-20 must occur from a "left-handed" to "right-handed" structure to generate the natural 20*R*-stereochemistry), followed by a second *covalent* attachment of a C-9 intermediate to generate 9β,19-cyclopropane ring stereochemistry. Neutralization of the charge generated at C-9 during the backward rearrangement of the C-20 cation then allows removal of the X^- group with formation of the cyclopropane ring by trans elimination. This mechanism gives the final bent product that is three-dimensionally different from lanosterol. (ii) The substrate assumes a prefolding for binding to the synthase that approximates the structure *trans-syn-trans-anti-trans-syn*-1,2-cyclopentanoperhydrophenanthrene, which generates a stable intermediate with a 17β-side chain and a 20*R*-stereochemistry (**3** in Figure 11). In our proposal, no discrete transition state necessarily forms to stabilize a C-20 carbocation (the basis of the X^--group mechanism), rather the ionic intermediate that involves the 17-(20)-bond (**2** in Figure 11) is a bridged carbenium ion which can promote intramolecular rearrangements with greater facility than a C_{20} positively charged intermediate. Alternatively, the stereochemical evidence regarding formation of loss of hydrogen from the C-19 methyl group (i.e., with retention of configuration)[34] to form a cyclopropane group indicates intermediates are formed on the reaction pathway during the terminal steps, as they apparently are formed at several steps during ring annulation. The steric course of the hypothetical reaction agrees with the solution and solid-state data showing that the molecule is flat. The labeling pattern of the product derived from appropriately isotopically labeled precursors indicates that the 8β-hydrogen is labeled,[3a,17b] suggesting that the 9β-hydrogen, abstracted during lanosterol formation, undergoes proton shuttling with the enzyme to provide the labeled hydrogen that is added to the sterol following nucleophilic substitution at C-19.

The origin of lanosterol in plants is generally thought to be from conversion of cycloartenol.[3] However, the putative enzyme-bound intermediate lanosterol has been observed directly from assays of radiolabeled squalene oxide using an alga

(29) For a key to the literature of molecular modeling of steroids of the sort described here, see: Wiese, T. E.; Brooks, S. C. J. *Steroid Biochem. Mol. Biol.* **1994**, *50*, 61.

(30) We have reported the solid-state structure of sitosterol in the following: Guo, D.; Venkatramesh, M.; Nes, W. D. *Lipids* **1995**, *30*, 203. Crystallographic data were obtained by J.F.G. (and W.D.N.) and are unpublished.

(31) Venkatramesh, M.; Nes, W. D. *Arch. Biochem. Biophys.* **1995**, *324*, 189.

(32) Kadota, S.; Li., J. X.; Tanaka, K.; Namba, T. *Tetrahedron* **1995**, *51*, 1143.

(33) (a) Cornforth, J. W. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 903. (b) Caspi, E. *Tetrahedron* **1986**, *42*, 3. See also refs 3a,b.

(34) (a) Altman, L. J.; Han, C. Y.; Bertolino, A.; Handy, G.; Laungani, D.; Mullaer, W.; Schwartz, S.; Shanker, D.; de Wolf, W. H.; Yang, F. *J. Am. Chem. Soc.* **1978**, *100*, 3235. (b) Independent findings for retention of configuration by D. Arigoni is cited in the latter paper.

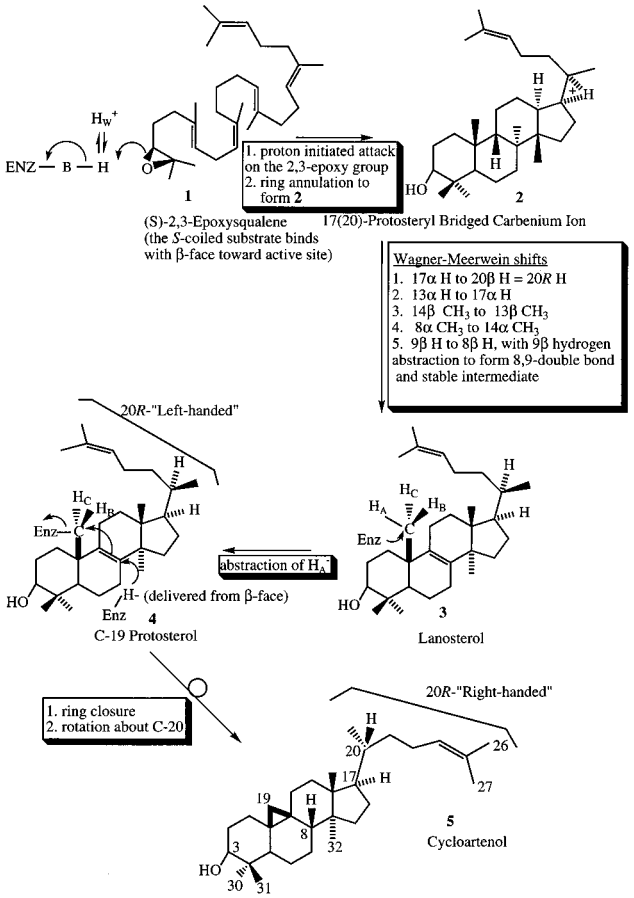

Figure 11. Hypothetical mechanism for the cyclization of squalene oxide to cycloartenol.

enzyme, and this may account for the fact that lanosterol is widely occurring in plants.[35,36] The side chain of the lanosterol-bound intermediate has the side chain on the left, for mechanistic reasons. Alternatively, the final product, that is free of the enzyme, has the side chain on the right, for conformational reasons. Thus typical substrate and intermediate analogues that possess "right-handed" side chains naturally would not be expected to productively bind to the synthase and hence would not trap or otherwise undergo transformation. It also follows that accumulation of cycloartenol or lanosterol (e.g., resulting from developmental regulation or by inhibitors)[30,37] will not act as a feedback regulator of squalene oxide cyclization to sterols. The hypothetical mechanism reported here for the cyclization of squalene oxide to cycloartenol raises the intriguing possibility that the lanosterol synthase (organisms with a nonphotosynthetic lineage) preceded the cycloartenol synthase (organisms with a photosynthetic lineage) in evolution and that rational design of agrochemicals and genetic engineering of synthase enzymes highly specific for the phytosterol pathway may be forthcoming.

(35) Giner, J.-L.; Wunsche, L.; Andersen, R. A.; Djerassi, C. *Biochem. System. Ecol.* **1991**, *19*, 143.

(36) Akihisa, T.; Kokke, W. C. M. C.; Tamura, T. In *Physiology and Biochemistry of Sterols*; Patterson, G. W., Nes, W. D., Eds.; American Oil Chemists' Society Press: Champaign, IL, 1992; p 172.

(37) We have shown that under normal growth conditions a fungus treated with 2,3-epiminosqualene accumulates squalene oxide, but the fungus will not accumulate squalene oxide upon treatment with 24,25-epiminolanosterol which causes accumulation of lanosterol. A similar trend was observed in the yeast-like alga *Prototheca wickhermaii* and cultured plants cells in which epiminolanosterol-treated cells induced accumulation of cycloartenol (cf. Janssen, G. G.; Kalinowska, M.; Norton, R. A.; Nes, W. D. In *Physiology and Biochemistry of Sterols* Patterson, G. W., Nes, W. D., Eds.; American Oil Chemists' Society Press: Champaign, IL, 1992; p 83.

Experimental Section

NMR Studies. Cycloartenol was isolated from γ-orazyanol and purified by reversed-phase HPLC. Solutions (0.1 M) were made up in $CDCl_3$ solution. ^1H and ^{13}C NMR measurements were carried out at 500 MHz (JEOL α-500) using an standard JEOL sequences for 1D and 2D NMR measurement. 2D phase-sensitive NOESY spectra were recorded with the mixing times set at 300 and 600 ms. HMBC spectra were parametrized to 5 and 8 Hz to favor long-range proton−carbon couplings. The spin−lattice relaxation time (T_1) was measured by using the inversion−recovery pulse sequence (RD−180°−τ−90°−acq) with a relaxation delay of 30 s and 64K data points on a JEOL 500 MHz at 30 °C. Each T_1 measurement was repeated at least three times, and its averaged values has an accuracy of ±10%. Chemical shifts are measured relative to TMS (δ, 0.0). All coupling constants are in units of hertz.

NMR Simulation. Calculated spectra were produced with the program from ref 39. The input δ and J values were read from 2D J spetrum, and the J values were adjusted for the best visual agreement with the observed subspectra.

Simulation Methodology. All calculations were performed using the MacroModel and BathchMin V5.0 molecular modeling programs (MacroModel V 5.0).[38] The initial minimization was carried out by Truncated Newton Conjugate Gradient method, the MM3* force field incorporated in MacroModel. Simulations were performed at 900 and 2000 K, respectively, using mixed Monte Carlo/stochastic dynamics (MC/SD) method. The total simulation time in all cases was 100 ps with a 0.75-fs time step for the SD part of the simulation algorithm. The simulation was carried out in stage, and the first 5 ps of each simulation was taken as an equilibration period and discarded. The MC part of the MC/SD algorithm used random torsional rotations between ±180° and was applied to $C_{17}-C_{20}$, $C_{20}-C_{22}$, $C_{22}-C_{23}$, and $C_{23}-C_{24}$ of the side chain. The ratio of SD steps to MC steps was 1:1, and the acceptance rates for MC part of the simulation was around 5%. Nonbonded cutoff distances were not used. Structures corresponding to high-energy geometries observed at 1-ps interval throughout the whole simulation step were saved for use as starting conformation for further optimization. Sampling intervals more frequently than 1 ps were not found to produce additional low-lying minima.

Force Field. The molecular mechanics force field used in these calculations was MM3*. All force field equations are identical with those of Allingers' authentic MM3 except for (i) the electrostatic equation (MM3* uses partial charges/Coulombs law, whereas MM3 uses bond dipoles/jeans equation); (ii) the out-of-plane bending equation (MM3* uses an improper torsion, while MM3 uses a pyramidalization distance); and (iii) the way conjugation is manipulated (MM3* uses specific V2 torsional terms for various conjugated system generally uses an SCF π calculation).

Evaluation of Simulation Convergence. Results obtained from molecular simulations are meaningful only if the simulations can be demonstrated to be converged, i.e., give results which are invariant of starting conditions and simulation length. One of the most convincing proofs of convergence is the demonstration that identical average properties can be obtained by starting simulations from very different initial conditions. To test for starting condition independence, we performed MC/SD simulations with cycloartenol starting from different conformations, flat and bent and with different simulation temperatures of 900 K and 2000 K. In both cases the MC/SD simulation gave the same global minima, the flat conformation. Simulation at 900 K found the most stable conformers eight times among 100 conformers sampled from MC/SD simulation while simulation at 2000 K gave the most stable conformer four times. Most interestingly, when the most stable conformer from high-temperature simulation is further subjected to MC/SD at 298 K, the simulation gave out the same conformer 20 times out of the 100 conformers sampled. The above evidence demonstrated that our simulation is well converged.

(38) Mohamadi, F.; Richards, N. J. G.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caulfield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440.

(39) Satake, K.; Hara, ZY.; Murara, H.; Yamamoto, Y. *Chem. Suppl.* **1984**, *3*, 1.

Energy Change with Rotation about the 17(20)-Bond. Input starting structure was the most stable global minimum from the above MC/SD simulation. The $C_{17}-C_{20}$ bond was rotated through 360° at 3° intervals and energy minimized by truncated Newton conjugate gradient method, MM3* force field.

Structure Determination. The crystals of both 31-norcycloartenol acetate ($C_{31}H_{50}O_2$) and 30,31-dinorcyloartenol acetate ($C_{30}H_{48}O_2$) were clear, colorless elongated prisms. A plate-shaped crystal of 30,31-norcycloartenol acetate, 0.05× 0.20 × 0.50 mm, was grown by slow evaporation from acetone/acetonitrile. Cell constants were determined by least-squares refinement of 45 reflections in the interval 15.80° < 2θ > 27.97°. Crystal data: $a = 13.799(4)$ Å, $b = 7.448(2)$ Å, $c = 13.767(4)$ Å, $\alpha = \gamma = 90°$, $\beta = 111.41(2)°$, space group = $P2_1$, $Z = 2$. Intensity data were collected on an Nicolet/Siemens P3 computer-controlled diffractometer using Nb-filtered Mo radiation ($\lambda = 0.710\ 73$ A) and $\theta-2\theta$ scans. The data were measured at 107 ± 0.5 K using a liquid nitrogen cold stream low-temperature apparatus. The data extended to (sin θ)/ $\lambda = 0.65$, 4475 reflections were measured, merging to 3287 unique reflections (2437 with $F_o > 2\sigma(F_o)$), and $R_{sym} = 0.03$. The structure was solved using MULTAN and NQEST. Final $R = 0.075$.

31-Norcycloartenol acetate, a plate-shaped crystal, 0.25 × 0.55 × 0.60 mm, was grown by slow evaporation from ethanol. Cell constants were determined by least-squares refinement of 45 reflections in the interval 20.04° < 2θ > 29.99°. Crystal data: $a = 13.821(2)$ Å, $b = 7.527(1)$ Å, $c = 13.809(2)$ Å, $\alpha = \gamma = 90°$, $\beta = 108.13(1)°$, space group = $P2_1$, $Z = 2$. Intensity data were collected on an Nicolet/Siemens P3 computer-controlled diffractometer using Nb-filtered Mo radiation ($\lambda = 0.710\ 73$ A) and $\theta-2\theta$ scans. The data were measured at 107 ± 0.5 K using a liquid nitrogen cold stream low-temperature apparatus. The data extended to (sin θ)/$\lambda = 0.75$, 5424 reflections were measured, merging to 4298 unique reflections (4040 with $F_o > 2\sigma(F_o)$), and $R_{sym} = 0.019$. The structure was solved using MULTAN and NQEST. Final $R = 0.045$.

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Supporting Information Available: Tables of atomic coordinates, position parameters, and thermal parameters and a list of 9β,19-cyclosterols from the Cambridge database (6 pages, print/PDF). See any current masthead page for ordering information and web access instructions.

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