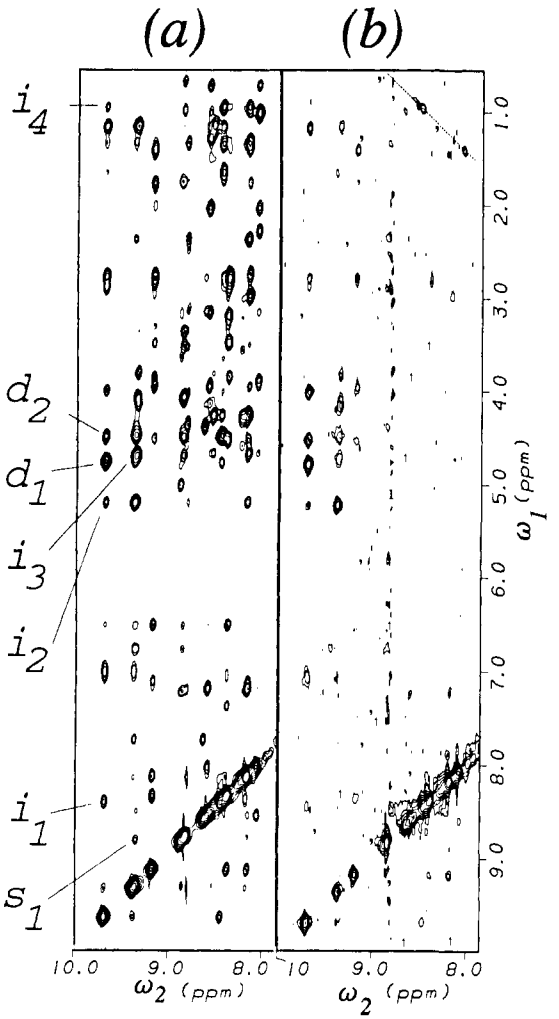

Figure 1. Cross-relaxation spectra (600 MHz) of ovomucoid third domain (OMTKY3) in ^2H_2O at pH* 4.1 and 25 °C. (a) Normal phase-sensitive NOESY spectrum;[15] (b) SNOESY spectrum obtained with pulse sequence II, with selective inversion of peaks in the 9.1–9.8 ppm range: 9.20 (Cys^{38} H^N), 9.37 (Ser^{26} H^N), 9.39 (Gly^{25} H^N), and 9.7 ppm (Tyr^{31} H^N).[16] Both spectra were base plane corrected[17] and scaled to a common intensity by reference to peak d_1, which is unaffected by spin diffusion as determined from cross-relaxation build-up curves.[4] Three classes of cross peaks are noted in the spectra: peaks d_1 and d_2 arise from direct magnetization transfer (d_1: Tyr^{31} H^N, Thr^{30} H^γ; d_2: Tyr^{31} H^N, Tyr^{31} H^α); peaks i_1–i_4 contain partial contributions from spin diffusion (i_1: Tyr^{31} H^N, Leu^{23} H^N; i_2: Tyr^{31} H^N, Cys^{24} H^α; i_3: Gly^{25} H^N, Gly^{25} H_1^α, Ser^{26} H^N, Gly^{25} H_1^α; i_4: Tyr^{31} H^N, Leu^{23} H^β); and peak s_1 arises from pure spin diffusion (s_1: Ser^{26} H^N, Ser^{51} H^N). One should note significant reduction of S/N in spectrum b. S/N deterioration is intrinsic to the method due to the dissipative character of magnetization transfer in the rotating frame.

changed (sequence II, Scheme II), then the second term in eq 2 becomes negative for s and all its neighbors:

$$\sigma_{ks}^{\mathrm{eff}} = \sigma_{ks}^{\mathrm{n}}\frac{\tau^{\mathrm{n}}}{\tau_{\mathrm{m}}} - \sigma_{ks}^{\mathrm{r}}\frac{\tau^{\mathrm{r}}}{\tau_{\mathrm{m}}} \qquad (3)$$

Under these conditions when $\tau^{\mathrm{n}} = 2\tau^{\mathrm{r}}$, one finds that $\sigma_{ks}^{\mathrm{eff}} = {}^4/_3\sigma_{ks}^{\mathrm{n}}$. Cross relaxation between the inverted resonance s and each of its neighbors is enabled, whereas direct cross relaxation between all other spins remains suppressed ($\sigma_{kl}^{\mathrm{eff}} = 0$).[12]

The direct cross-relaxation and spin-diffusion pathways of the small protein, turkey ovomucoid third domain (OMTKY3, 6062 Da), have been well documented.[13] When a long mixing time is used, most of the NOESY cross peaks (Figure 1a) contain contributions from spin diffusion. By contrast, all of the cross peaks in the SNOESY spectrum (Figure 1b) obtained with the same mixing time (199 ms) arise solely from direct magnetization transfer.[14] For example, peaks d_1 and d_2 (Figure 1a), which are due to pure, direct magnetization transfer, have the same amplitude in both spectra; peaks i_1–i_4, which have contributions from both direct and indirect cross relaxation in the NOESY spectrum, are reduced in amplitude in the SNOESY spectrum, and peak s_1 (Figure 1a), which arises from pure spin diffusion, is absent in the (9.1–9.8 ppm) SNOESY spectrum (Figure 1b). The above results are consistent with our previous analysis of cross relaxation in OMTKY3.[4,6]

The DNOESY[6] pulse sequence removes spin-diffusion effects over the entire spectrum but fails in regions where diagonal peaks overlap. By contrast, SNOESY fails only in regions where diagonal peaks *from spatially close neighbors* overlap. SNOESY requires that separate data sets be obtained for each spin. This requirement might be relaxed by a suitable heteronuclear edited mixing sequence with an ^{15}N- or ^{13}C-labeled protein.

Acknowledgment. This study was carried out at the National Magnetic Resonance Facility at Madison, WI, under support from NIH Grants LM04958 and RR02301. Equipment in the facility was purchased with funds from the University of Wisconsin, the NSF Biological Instrumentation Program (Grant DMB-8415048), the NIH Biomedical Research Technology Program (Grant RR02301), the NIH Shared Instrumentation Program (Grant RR02781), and the U. S. Department of Agriculture.

(12) G. Bodenhausen (4th Chianti Workshop on Magnetic Resonance: Nuclear and Electron Relaxation, San Miniato (Pisa), Italy, June 2–8, 1991) has proposed an elegant experiment in which direct cross relaxation (free of spin diffusion) is observed between a *single pair* of spins within a multispin system. By contrast, the experiment demonstrated here provides all direct cross relaxations from a selected spin (or group of spins).

(13) Fejzo, J.; Zolnai, Z.; Macura, S.; Markley, J. L. *J. Magn. Reson.* **1990**, *88*, 93.

(14) When more than one spin is inverted, the spectrum can contain spin-diffusion cross peaks between pairs of inverted spins if they share a common cross-relaxation partner.

(15) Transients (32) were digitized with 2048 t_2 data points for each of 512 t_1 increments; τ_{m} = 199 ms.

(16) Transients (80) were digitized with 2048 t_2 data points for each of 512 t_1 increments: τ_{m} = 199 ms; τ^{r} = 22.1 ms; τ^{n} = 37.7 ms; the number of cycles, k = 3. A selective 180° Gaussian pulse (t_p = 3.23 ms) was applied over the 9.1–9.8 ppm range. Direct cross-relaxation cross peaks appear in both ω_1 and ω_2 only from resonances within this window.

(17) Zolnai, Zs.; Macura, S.; Markley, J. L. *J. Magn. Reson.* **1989**, *82*, 496.

The Methyl Group at C(10) of 2,3-Oxidosqualene Is Crucial to the Correct Folding of This Substrate in the Cyclization–Rearrangement Step of Sterol Biosynthesis

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Recently, much new information has been obtained regarding the molecular details of the cyclization–rearrangement step in sterol biosynthesis from 2,3-oxidosqualene (**1**), including the nature of the protosterol intermediate (**2**) and the manner in which the stereochemistry at C(20) is rigorously controlled.[1-3] Described herein are new data and insights on the interesting question of how lanosterol synthase controls the stereochemistry of the ring system during the cyclization **1** → **2**, especially with regard to the generation of the high-energy B-ring twist-boat structure inherent in the trans-syn-trans geometry of the A/B/C ring portion of **2** (Chart I).

Studies carried out in this laboratory more than 20 years ago showed that the lanosterol synthase from hog liver converts the

(1) Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1990**, *112*, 6429–6431.
(2) Corey, E. J.; Virgil, S. C. *J. Am. Chem. Soc.* **1991**, *113*, 4025–4026.
(3) Corey, E. J.; Virgil, S. C.; Sarshar, S. *J. Am. Chem. Soc.* **1991**, *113*, 8171–8172.

0002-7863/92/1514-1524$03.00/0

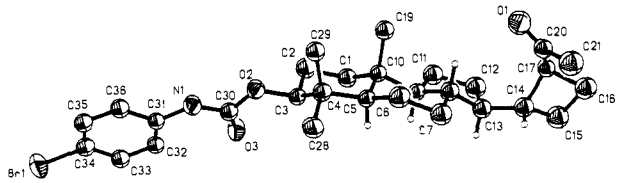
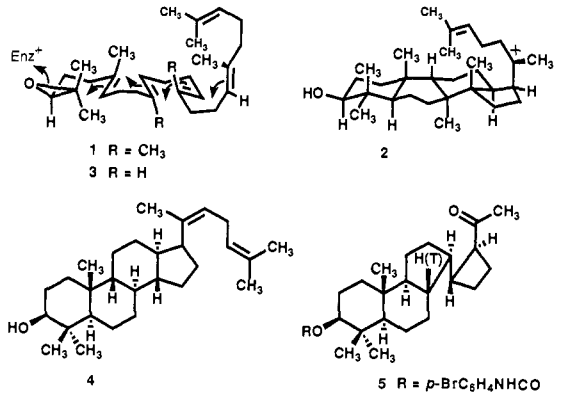


Figure 1. X-Ray structure of (*p*-bromophenyl)urethane **5**. Hydrogen atoms have been included at C(5), C(8), C(9), C(13), and C(14) in order to indicate the stereochemistry at these positions.

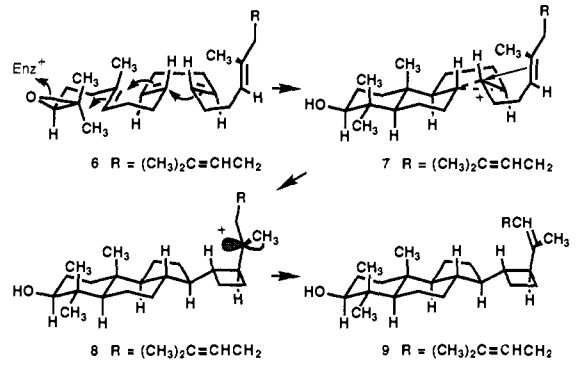
Chart I



oxidosqualene analogue **3**, lacking the methyl substituents at C(10) and C(15) of 2,3-oxidosqualene, to a product of cyclization *without* rearrangement of carbon and hydrogen.[4] Since only microgram amounts of the enzymic product from **3** were available, a rigorous determination of structure was not possible. However, structure **4** emerges as a possibility if a normal cyclization pathway is assumed.[4] We show herein that this assumption is not valid and that this enzymic cyclization is even more revealing than previously thought.

Tritium-labeled **3**[4,5] was incubated with large amounts of the microsomal protein from hog liver[6] to afford a labeled cyclization product (14.4% radiochemical yield) having the same R_f as lanosterol (0.36 with 1:1 ether–hexane on silica gel (sg) plates). After sg column chromatography, the product was converted to the *p*-bromobenzoate derivative. Ozonolysis of the *p*-bromobenzoate (−78 °C, CH_2Cl_2–CH_3OH, Sudan red III) and purification by sg HPLC afforded a crystalline methyl ketone. Although this derivative (ca. 4 mg) was pure by HPLC and 500-MHz ^1H NMR analysis, the crystals obtained from many solvents were twinned and not suitable for X-ray analysis. Because the methyl ketone was not epimerized by base, the corresponding (*p*-bromophenyl)urethane could be prepared by saponification of the *p*-bromobenzoate (LiOH, aqueous THF) and reaction with (*p*-bromophenyl)isocyanate. Clear prisms of (*p*-bromophenyl)-urethane, mp 229–231 °C, were grown from a dichloromethane–hexane bilayer at 23 °C and subjected to X-ray crystallographic analysis, which revealed the structure to be **5**; see Figure 1.[7] The structure of the cyclization product from **3** is therefore **9**.

Scheme I



The formation of **9** can be explained[2,3] in terms of the following process: (1) preorganization of **3** on the enzyme in conformation **6**, (2) electrophile-induced cyclization via **7** and **8** and deprotonation to **9** (Scheme I). The folding of the substrate shown in **6** differs from that for the cyclization of 2,3-oxidosqualene to form lanosterol by a rotation of ca. 180° of the C(10)–C(11) olefinic subunit. The folding geometry of the other parts of **6** in three dimensions and the overall shape are close to that involved in the biocyclization of **1** to **2**. This leads to an important question: Why does the cyclase allow **3** to adopt the geometry shown in **6** with a β-oriented C(10) H, whereas **1** is constrained to fold with the α-orientation of the C(10) methyl group (α and β are used herein the sense of the steroid convention)? A possible answer is that there is a hydrophobic group of the cyclase which is at van der Waals contact with the 10β H of **6** and which is large enough to prevent **1** from assuming the same folding because of a strong repulsion with a 10β methyl group in this geometry. This key group on the enzyme may well be a methyl group of leucine, isoleucine, or valine.[8]

Although cation **7** could undergo ring closure to form a 6,6,6,5-fused ring system, this mode of cyclization, which would give an isomeric protosterol having trans-anti-trans-syn-trans geometry with a twist-boat C-ring, is evidently not favorable relative to formation of a 5-membered C-ring. Generation of the 6,6,5-fused A/B/C system then leads to the unusual Markovnikov cyclization to form the cyclobutylcarbinyl cation **8**, the deprotonation of which by the enzyme must be fast compared with rearrangement.

We have not been able to obtain **9** or any other cyclization product from **3** using the lanosterol synthase from yeast.[2,3] Indeed, we have determined from kinetic studies that **3** is a very effective time-dependent *irreversible* inhibitor of the yeast cyclase. This enzyme-induced irreversible inactivation is also observed with the hog liver cyclase, but is considerably less efficient. Consequently, it is possible to effect the conversion of **3** to **9** by using a large quantity of the hog liver enzyme. It is tempting to speculate that the inactivation of lanosterol synthase by **3** may be due to attack by cation **8** on sensitive nucleophilic groups of the enzyme which are within range of this rotationally flexible structure.

In summary, we have observed a unique diversion of the enzymic pathway of sterol biocyclization which is the result of a simple replacement of hydrogen for methyl of oxidosqualene and which reveals a critical feature of enzymic control of this fascinating process.[9]

Supplementary Material Available: Listings of crystal data, atomic coordinates, bond distances and angles, and thermal parameters for (*p*-bromophenyl)urethane **5** (14 pages); listing of observed and calculated structure factors for **5** (19 pages). Ordering information is given on any current masthead page.

(4) Corey, E. J.; Ortiz de Montellano, P. R.; Yamamoto, H. *J. Am. Chem. Soc.* **1968**, *90*, 6254–6255.

(5) Synthetic **3** containing tritium at C(10) was synthesized by a route different from that previously described,[4] but using well-known methods to be described separately. The structure was fully confirmed by analytical data, including the 500-MHz ^1H NMR spectrum.

(6) Substrate **3** labeled with tritium at C(10) (110 μmol, 50 × 10^6 cpm) was solubilized with 160 mg of Tween 80 detergent and incubated anaerobically with 250 g of hog liver microsomal protein suspended in 900 mL of 0.1 M pH 7.4 potassium phosphate buffer at 23 °C for 30 h.

(7) A total of 10338 reflections were collected at room temperature from a single crystal of **5** (0.2 mm × 0.5 mm × 0.7 mm) belonging to the orthorhombic $P_{2_12_12_1}$ space group with unit cell dimensions a = 10.993 (2) Å, b = 23.104 (3) Å, c = 11.005 (2) Å, β = 104.49 (2)°, Z = 4. Least-squares refinement of the data using 3136 reflections converged upon the structure shown in Figure 1 with R = 0.0441 and a goodness-of-fit = 0.70.

(8) Our analysis indicates that the key hydrophobic groups of lanosterol synthase which are responsible for the precise contacts and closeness of fit required to ensure proper folding of 2,3-oxidosqualene for the conversion **1** → **2** may also be the methyl groups of Leu, Ile, and Val. Methyl groups have the proper size for precise interdigitation with folded **1**.

(9) We are grateful to the National Science Foundation and the National Institutes of Health for generous financial support and to Mr. Seiichi P. T. Matsuda for helpful advice and discussion.