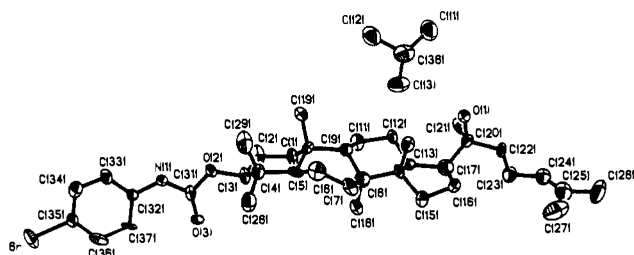

Figure 1. Structure of (*p*-bromophenyl)urethane **8** as $CHCl_3$ solvate (grown from a $CHCl_3$–isooctane bilayer at 4 °C) as determined by X-ray diffraction. Selected distances (Å): C(20)–C(21) = 1.527; C(20)–O(1) = 1.444.

°C and pH 7 for 12 h gave, after chromatographic purification, the same product (**5**) in 14% isolated yield (0.35 mg).

Reaction of the 3-*tert*-butyldimethylsilyl ether of **1**, which is available from either total synthesis[2,5] or biosynthesis,[2] with the Grignard reagent $Me_2C{=}CHCH_2CH_2MgBr$ in THF at 0 °C for 3 h produced **6** with 9:1 diastereopreference over the more polar C(20) diastereomer.[6] Conversely, the C(20) diastereomer of **6** was the major product (ca. 9:1 diastereoselection) of the reaction of CH_3MgBr with the ketone corresponding to the replacement of CH_3CO in **1** by $(CH_3)_2C{=}CHCH_2CH_2CO$. The proof of structure of **6** was effected by desilylation to **7** and conversion to the (*p*-bromophenyl)urethane **8**, whose structure was determined by single-crystal X-ray diffraction analysis to be as shown in Figure 1.[7] Diols **5** and **7** were interrelated by conversion to identical samples of diol **9**[8] and silyl ether **10**.[8]

The finding that **2** is converted to the protosterol derivative **5** by the sterol cyclases has several mechanistic implications: (1) Closure of the D ring generates a protosterol having the 17β side chain.[1] (2) Water attaches preferentially to C(20) of the tetracyclic C(20)–C(25) pentadienyl cation from **2** (**11**) at a rate which is fast compared to rearrangement of hydrogen from C(17) to C(20).[9] (3) In the biocyclization of **2** to form **5** the addition of carbon and HO to the C(18)–C(19) double bond of **2** is stereospecific and antarafacial, implying that the attachment of water to C(20) in **11** is fast relative to C(17)–C(20) bond rotation. (4) The position-specific attachment of water to cation **11** at C(20) suggests that C(23) and C(25) may be in a binding pocket which shields them from nucleophilic attack. (5) The enzyme-associated water which converts **11** to **5** may stabilize the protosterol C(20) cation from 2,3-oxidosqualene (**12**), but does not react with it at a rate which is appreciable relative to hydride migration from C(17) to C(20).

The β-orientation of the side chain at C(17) and the strong steric interaction with the cis 14β-methyl substituent clearly serve to hinder rotation about the C(17)–C(20) bond in **11** and thereby favor stereospecific attachment of hydroxyl to C(20). The β-orientation of the side chain at C(17) also facilitates the control of configuration at C(20) in lanosterol biosynthesis from 2,3-oxidosqualene, since the protosterol cation **12** is generated in the correct geometry for C(17) → C(20) hydride migration and since C(17)–C(20) bond rotation is restricted. Dramatic support for this idea has been obtained from experiments on the chemical rearrangement of **13**, the 24,25-dihydro-3-benzoate of **7**, and the C(20) diastereomer of **13**. Reaction of **13** with BF_3 in CH_2Cl_2 at −90 °C for 3 min produced 24,25-dihydroparkeol benzoate (**14**) (90%) stereoselectively, and similarly, the C(20) epimer of **13** gave the C(20) epimer of **14** (90%). In these rearrangements there is overall retention of configuration in the replacement of the C(20) hydroxyl by hydrogen. The reaction of **13** is considered to occur by (1) complexation of BF_3 with the (thermodynamically less stable) conformation shown in formula **13**, (2) heterolysis to **15**, and (3) rearrangement of hydrogen from C(17) to C(20) at a rate which is fast compared to the restricted rotation of the C(17)–C(20) bond.[10] In contrast, as noted earlier,[5] the BF_3-catalyzed rearrangements of the 17α-epimer of **13** and its C(20)-epimer are nonstereoselective at C(20), each giving a 1:1 mixture of C(20) epimers of dihydroparkeol benzoate.[5] It is clear that in the enzymic cyclization of 2,3-oxidosqualene the 17β protosterol pathway via cation **12** is superior to the 17α protosterol pathway with respect to stereospecific production of the natural 20*R* configuration of sterols.[1,11]

Supplementary Material Available: Experimental procedures and spectroscopic data for compounds **2**, **5–10**, **13**, 20-*epi*-**6**, 20-*epi*-**9**, **14**, and 20-*epi*-**14** and a listing of crystal data, atomic coordinates, bond distances and angles, and thermal parameters for (*p*-bromophenyl)urethane **8** (35 pages); listing of observed and calculated structure factors for **8** (29 pages). Ordering information is given on any current masthead page.

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(6) R_f values for **6** and the C(20) diastereomer on silica gel plates (10% ether–hexane) were 0.29 and 0.25, respectively.
(7) The conformation about the C(17)–C(20) bond of **8** as shown in Figure 1 corresponds to that which is expected to be most stable.
(8) As shown by chromatographic and spectroscopic comparison.
(9) The stabilization of the pentadienyl cation subunit in **11** relative to a localized C(20) cation probably diminishes the rate of H migration from C(17) to C(20) by several orders of magnitude.
(10) Although the conformation shown in Figure 1 is clearly more stable than the C(17)–C(20) rotamer shown in **13**, the hydroxyl group at C(20) is much more accessible sterically in **13**.
(11) This research was assisted by grants from the National Institutes of Health and the National Science Foundation. We are indebted to Mr. Seiichi P. T. Matsuda for valuable information and help on the purification of the yeast cyclase.

Purification of the 2,3-Oxidosqualene-lanosterol Cyclase from *Saccharomyces cerevisiae*

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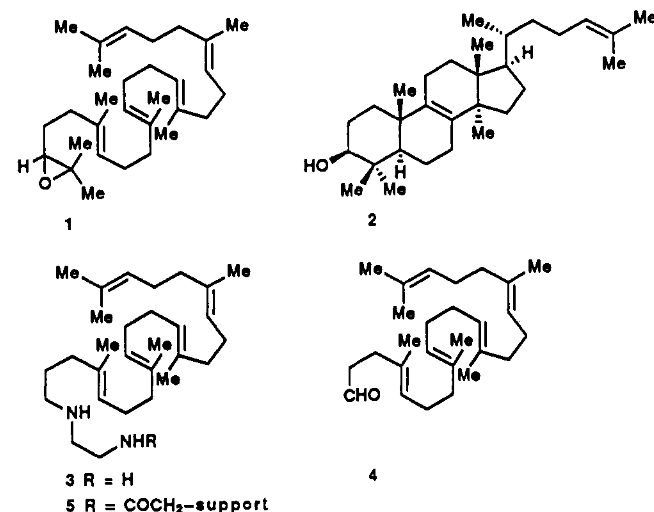
Probably the most remarkable step in the biosynthesis of cholesterol is the conversion of 2,3-oxidosqualene (**1**) to lanosterol (**2**) in a single supremely effective step by the enzyme 2,3-oxidosqualene-lanosterol cyclase EC 5.4.99.7 ("sterol cyclase").[1]



In contrast, a sequence of 18 additional steps is required to remove the three extraneous angular methyl groups of **2** and to generate

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Table I. Purification and Recovery[a]

fraction	vol, mL	protein mg/mL mg/mL	total protein, mg	act./mL, unit/mL	sp act., units/mg	purifn., -fold	recovery, %
microsomes	180	12	2160	0.34	28	1	100
DEAE I	180	0.8	144	0.28	350	12	82
DEAE II	120	0.17	20.4	0.32	1900	68	63
affinity	47	0.05	2.4	0.22	4400	160	17

[a] One unit is defined as the amount of enzyme that will convert 1 mg of oxidosqualene to lanosterol in 1 h. Protein was determined using the Bio-Rad assay according to the manufacturer's directions.

the Δ^5-olefinic linkage of cholesterol. Because of the unsurpassed effectiveness and control inherent in the cyclization of **1** to form **2**, we have for some time been interested in obtaining the sterol cyclase in pure form for X-ray crystallographic studies with a bound substrate-based inhibitor, e.g., 2,3-iminosqualene.[2] Progress in the purification of the sterol cyclase[3-6] has been retarded by its membrane-bound nature, its instability, and its insolubility in the absence of detergents. We report herein a solution to this difficult and longstanding problem using the enzyme from yeast (*Saccharomyces cerevisiae*)[2] and a rationally designed affinity matrix as a crucial step.

Dry bakers' yeast (Sigma, 80 g) was hydrated in 500 mL of H_2O for 30 min. Cells were collected by centrifugation [15 min at $(5 \times 10^3)g$] and washed twice with 500 mL of H_2O and once with 500 mL of 100 mM sodium phosphate, pH 7.0. The hydrated yeast (160 g) was resuspended (40% w/v) in the same buffer at 0 °C, the cells were lysed by two passages through a French press (20 000 psi, 4 °C), and the effluent was immediately cooled to 0 °C and then centrifuged (30 min at 10^4g) to sediment unlysed cells and cell debris. Microsomes were sedimented from 160 mL of the 10^4g supernatant in a Beckman ultracentrifuge (2 h at 10^5g at 4 °C in a 70 Ti rotor). The microsomes were freed of peripheral proteins by being resuspended in 160 mL of 500 mM sodium phosphate, pH 6.2, and then sedimented again (1 h at 10^5g). The washed microsomes were solubilized in 160 mL of 1% Triton X-100 using a Dounce homogenizer, and the insoluble material was sedimented (1 h at 10^5g).

The solubilized enzyme of the supernatant was adjusted to pH 7.0 (*critical*) using 1 M sodium hydroxide and applied to a DEAE-Sepharose column (50-mL bed volume) at 4 °C preequilibrated with 20 mM pH 7.0 sodium phosphate buffer containing 20% glycerol and 1% Triton X-100. The column was eluted with a linear gradient of pH 7.0 sodium phosphate buffer containing 20% glycerol and 0.2% Triton X-100 (600 mL from 20 to 100 mM). The activity eluted at 60–70 mM sodium phosphate in a volume of 150 mL.

The active fraction from DEAE-Sepharose chromatography was diluted with 2 volumes of 20% glycerol and 0.2% Triton X-100 to lower the phosphate concentration to 20 mM. The solution was applied to a 20-mL DEAE-cellulose column at 4 °C preequilibrated with 20 mM pH 7.0 sodium phosphate buffer containing 20% glycerol and 0.2% Triton X-100. A 600-mL linear gradient from 20 mM sodium phosphate, pH 7.0, containing 20% glycerol and 0.2% Triton X-100 to 200 mM sodium phosphate, pH 6.2, containing 20% glycerol and 0.2% Triton X-100 eluted the activity at pH 6.7.

A 20-mL aliquot of the active fraction from the preceding separation was diluted with 2 volumes of 500 mM pH 7.0 sodium phosphate buffer and applied to a 3-mL affinity column (at 23 °C) prepared as described below and previously equilibrated with 500 mM pH 7.0 sodium phosphate. The column was washed with 30 mL of 500 mM pH 7.0 sodium phosphate buffer containing 20% glycerol and 0.2% Triton X-100. Bound activity was eluted with 10 mL of 100 mM pH 4.0 sodium phosphate. Each 3-mL acidic fraction was collected into 1 mL of 500 mM pH 7.0 phosphate buffer in order to effect rapid neutralization. The pH 4.0 eluent contained a single protein with a molecular weight of ~26 kDa as estimated by SDS-PAGE,[7] which cleanly effected the conversion of 2,3-oxidosqualene to lanosterol[8] (Figure 1).

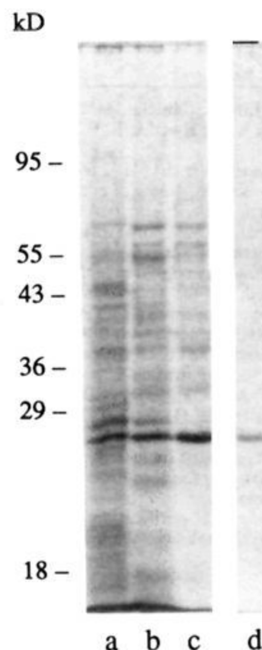

Figure 1. SDS–PAGE showing protein present after each purification step: (a) washed microsomes, (b) DEAE-Sepharose, (c) DEAE-cellulose, (d) affinity chromatography. Positions of molecular weight markers (Diversified Biotech) are shown at left.

The following approximate amino acid composition was measured for this cyclase by hydrolysis and amino acid analysis using HPLC (excluding cys and trp, which were not determined): Asx (30), Glx (31), Ser (22), Gly (26), His (7), Arg (10), Thr (15), Ala (17), Pro (10), Tyr (6), Val (11), Met (3), Ile (9), Leu (14), Phe (7), Lys (20). Attempted determination of partial sequence at the N-terminal end by the Edman method was inoperable, indicating that the terminal amino function is probably acetylated, in common with many other yeast enzymes.

The affinity column was prepared by amide formation between 25 mL of Affigel-15 (an activated ester available from Bio-Rad

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(8) Oxidosqualene-lanosterol cyclase activity was monitored by thin-layer chromatography. To 50 μL of enzyme was added 2.5 μL of aqueous 2,3-oxidosqualene solution (which contained 20 mg/mL (±)-2,3-oxidosqualene and 200 mg/mL Triton X-100 (w/v), giving a final concentration of 1 mg/mL oxidosqualene and 1% Triton X-100 after addition to enzyme). After 8–12 h at 25 °C, 5 μL of the aqueous reaction mixture was spotted on a TLC plate. Water was removed under vacuum, and the plate was developed in ether to 1 cm. The plate was then fully developed with hexane/ether (1:1). Oxidosqualene and lanosterol have R_f values of 0.91 and 0.54, respectively. Quantitative assays were performed identically, except for the following changes. The oxidosqualene was radioactive (5000 cpm/assay), and the assay was terminated after 1 h. The entire 50-μL incubation was applied to the TLC plate, and after chromatography, the areas corresponding to oxidosqualene and lanosterol were added to scintillation cocktail and counted.

Co.) and 200 mg of diamine **3** [prepared from trisnorsqualene aldehyde (**4**)[9] and ethylenediamine][10] in 2 mL of ethanol at 23 °C for 12 h, filtration, and washing with 250 mL of ethanol followed by 250 mL of distilled, deionized water. The resulting affinity matrix **5**, which was chosen because it retains both the H bond accepting capabilities and the C(4) → C(30) hydrophobic structure of the normal substrate **1**, is the first such reagent to function effectively in the purification of the sterol cyclase.

The purified sterol cyclase can be stored for at least 2 weeks at 0 °C without significant loss of activity. We believe that the way is now clear for the cloning of the gene for the yeast sterol cyclase and for the eventual determination of its structure.[11]

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(10) To 1.00 g of trisnorsqualenal[9] (**4**) (2.60 mmol) in 260 mL of anhydrous methanol at 0 °C was added 1.56 g of ethylenediamine (26.0 mmol, 10 equiv). Concentrated hydrochloric acid (0.43 mL, 5.2 mmol) was added, followed by 327 mg of sodium cyanoborohydride (5.2 mmol) in one portion. After 2 h at 0 °C, the methanol was removed in vacuo, and the residue was taken up in 200 mL of hexane and 300 mL of 1 M sodium bisulfate. The hexane layer was removed, and the aqueous layer was washed with another 200-mL portion of hexane. The aqueous layer was brought to pH 13 with solid sodium hydroxide and extracted with 3 × 200 mL of ether. The combined ether fractions were washed once with brine and then dried with anhydrous sodium sulfate. Concentration afforded 840 mg (76%) of monoalkylated diamine **3**: 400-MHz ^{1}H NMR ($CDCl_3$) δ 1.60 (s, 12 H), 1.68 (s, 3 H), 1.72 (s, 3 H), 2.04 (m, 18 H), 2.07 (t, 2 H, J = 7.1), 2.59 (t, 2 H, J = 7.4), 2.67 (t, 2 H, J = 5.8), 2.81 (t, 2 H, J = 6.1), 5.14 (m, 5 H).

(11) This research was assisted financially by a grant from the National Institutes of Health. We are grateful to Prof. Konrad Bloch for encouragement and helpful discussions and to Prof. A. Ian Scott for a preprint[5] describing a procedure for partial purification of the sterol cyclase and the optimal pH (6.2) for enzyme stability. We are indebted to William S. Lane for the amino acid analysis.

Mirror-Image DNA

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D-Deoxyribose is the only asymmetric unit of DNA. Chiral molecules have two possible enantiomers, possibly possessing the same chemical and physical properties, although the energy difference between the enantiomers, particularly at a nuclear physics level, is quite small.[1] The features of single-stranded L-oligonucleotides have been reported.[2] However, whether the duplex structure of a DNA composed of L-deoxyribose is also an exact mirror image of the natural one has yet to be determined. Thus, a conformational study was conducted on the self-complementary hexanucleotide d(CGCGCG) composed of L-deoxyribose.

L-Deoxycytidine was synthesized via the L-deoxyuridine derivative by glycosylation[3] of silylated uracil with 1-chloro-2-deoxy-3,5-di-*O*-toluoyl-L-*erythro*-pentofuranose (prepared from L-arabinose[4]) and subsequent conversion to L-deoxycytidine.[5] L-Deoxyguanosine[6] was synthesized by direct glycosylation as

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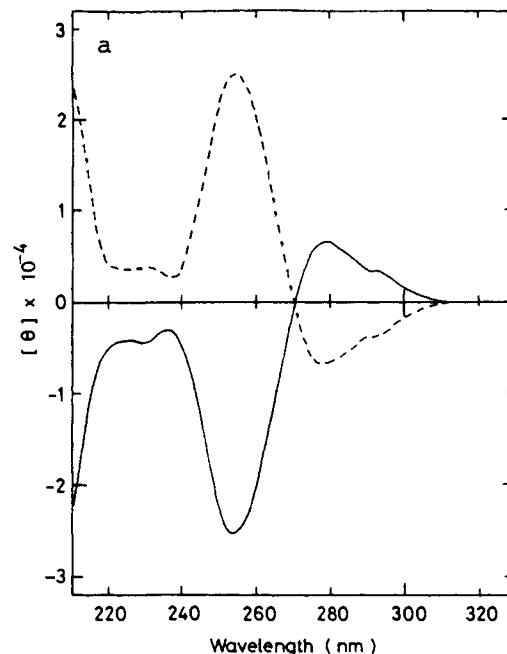

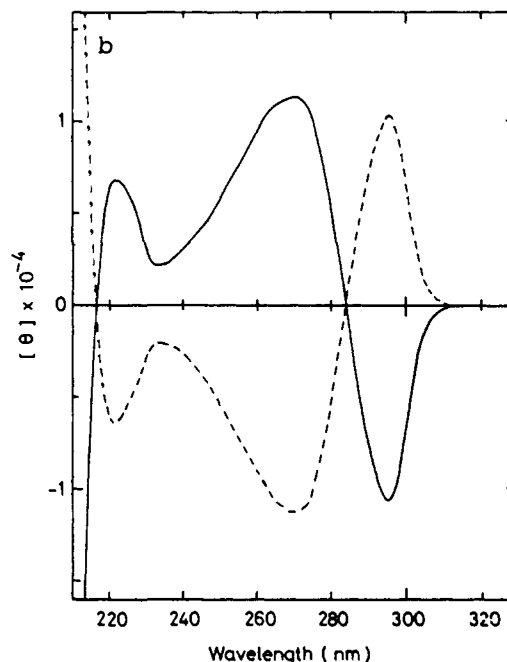

Figure 1. Circular dichroism spectra of D-d(CGCGCG) (solid line) and L-d(CGCGCG) (dashed line) at 0 °C. Samples (10 A_{260}/mL) were dissolved in 10 mM sodium phosphate buffer (pH 7) containing (a) 0.1 M NaCl and (b) 4 M NaCl. Strand concentration was estimated by absorbance values at 80 °C and a molar extinction coefficient of 51 400 L/(mol·cm) at 260 nm calculated for D- and L-d(CGCGCG).[12]

described by Robins et al.[6,7] L-Deoxynucleosides were protected in the usual way, and D- and L-d(CGCGCG) were synthesized by the β-cyanoethylphosphoramidite method.[8]

On reversed-phase HPLC, the retention time of L-d(CGCGCG) was consistent with that of the corresponding natural D-hexamer synthesized as a control. The purity of both hexamers was more than 95%. The enzymatic digestion of D-d(CGCGCG) with

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