complex cellular chain processes with a structure that, from the chemical point of view, is much simpler than a cell itself. The other aspect is the possibility of utilizing this approach for biotechnological purposes, i.e., for the production of PC.

The self-replication observed with the short-chain PC reveals the well-known extreme sensitivity of the supramolecular aggregate to the fine details of the chemical structure of the monomers: It also shows the versatility and tunability of these macromolecular complexes.

The self-replication is an important chemical process per se, as it provides a relatively simple synthetic system to mimick a basic cellular process. The relevance of this observation is made more stringent by the report that liposomes can be seen as prebiotic cells.[26,27]

To the best of our knowledge, no geometrically closed structures able to self-replicate have been thus far reported, except our micellar systems.[12] With respect to reverse micelles, liposomes offer the advantage of being closer to cells, regarding both their structure and the environment in which they operate. The fact that we are dealing with structures having a boundary that is itself constructed by the network of reactions taking place within the boundary itself permits to propose them as autopoietic units.[10,28] Autopoiesis can be defined as an operational definition of the living. The challenge in the liposome self-replicating system lies in the testing of this notion.

Acknowledgment. We thank Dr. L. F. J. Dürselen, who enabled the performance of all reactions with radioactive substances. Further we thank Prof. Dr. H. Zuber for kindly making available the super- and ultraspeed centrifuges.

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Mechanistic Studies on a Placental Aromatase Model Reaction

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Abstract: Aromatase is a cytochrome P-450 enzyme that converts androgens to estrogens via three successive oxidative reactions. The mechanism of the third step has previously been intensively studied, with no clear resolution. A leading theory for the third step proposes nucleophilic attack of the heme ferric peroxide species on the 19-aldehyde intermediate to produce a 19-hydroxy 19-ferric peroxide intermediate. We have shown previously that analogues of this intermediate failed to aromatize under nonenzymatic conditions. In this study, we prepared a 2,4-dien-3-ol analogue of the 19-aldehyde intermediate and showed that it reacted with HOOH to produce the corresponding estrogen derivative. Evidence has been accrued to suggest that this reaction, which we have called the aromatase model reaction, involves a 19-hydroxy 19-hydroperoxide intermediate. The model reaction was shown to be faithful to the actual aromatase-catalyzed reaction with regard to stoichiometric formic acid production, ^{18}O-incorporation patterns, and stereoselectivity for 1β-hydrogen removal. A kinetic analysis at 37 °C was also performed, and the reaction was demonstrated to be pseudo-first-order by using an excess of HOOH, and first-order with respect to HOOH at the concentrations studied. The effects of KOH, EDTA, and BHT on the reaction were also examined, and are discussed.

Placental aromatase is a cytochrome P-450 enzyme complex that catalyzes the conversion of steroidal androgens (testosterone (**1a**), androstenedione (**1b**)) to steroidal estrogens (estradiol (**4a**), estrone (**4b**)). The mechanism of aromatase has received widespread interest, in part because aromatase is a potential therapeutic target for selective lowering of estrogen levels in patients with estrogen-dependent tumors, including breast cancer.[1] Presumably elucidation of the aromatase mechanism could lead to the development of specific inhibitors. Furthermore, at a more fundamental level, the chemical reaction catalyzed by aromatase is complex and interesting and serves as a difficult challenge to mechanistic enzymologists.

Many aspects of the aromatase reaction have already been uncovered (see Figure 1). One human cytochrome P-450 protein[2] in conjunction with a nonspecific NADPH-dependent reductase is apparently responsible for effecting the entire conversion. Testosterone (**1a**), androstenedione (**1b**), and 16α-hydroxytestosterone are all direct substrates for the enzyme with similar values for k_{cat} but different values of K_m. Two successive hydroxylations at the angular 19-methyl group lead to intermediate 19-hydroxy **2** and 19-oxo **3** compounds. Both reactions are thought to be classical cytochrome P-450 type hydroxylations, with 1 equiv of molecular oxygen and NADPH consumed in each step.[3] Both reactions are stereospecific, the first occurring with retention of configuration and the second involving loss of the 19 pro-R hydrogen. Interestingly, there is a significant kinetic isotope effect in the first step but none in the second.[4a] Only the first oxygen equivalent consumed is incorporated into the aldehyde, suggesting a stereospecific dehydration of the presumed *gem*-diol intermediate. Elegant studies, by Covey and co-workers, on the processing by aromatase of false substrates suggest that active-site groups direct this dehydration.[4b]

In the third and last step, another oxidation is thought to occur with consumption of a third equivalent of molecular oxygen and NADPH.[5] The 1β-hydrogen is lost to the aqueous medium[6] and

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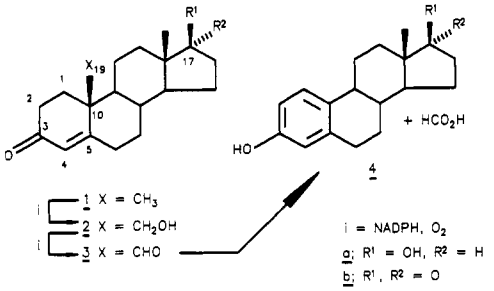

Figure 1.

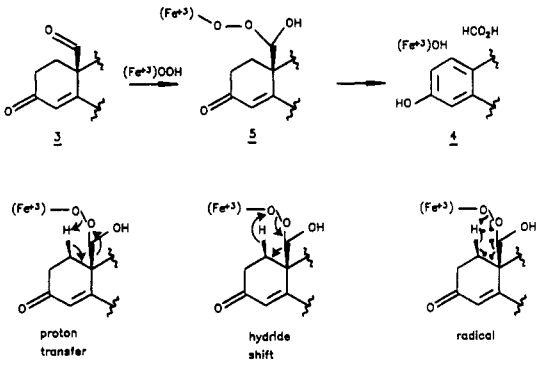

Figure 2.

carbon-19 is incorporated, along with one of its original hydrogens, into formic acid. Surprisingly, the stereoselectivity of removal of the 2β-hydrogen has been found to be substrate-dependent. While 19-oxoandrostenedione (**3b**) shows highly stereoselective loss (11:1) of the 2β-hydrogen, 19-oxotestosterone (**3a**) undergoes ca. 1:1 2α- and 2β-hydrogen loss.[7] This substrate-dependent stereoselectivity has been interpreted as indirect evidence supporting the importance of an active-site base in 2-hydrogen removal. The third equivalent of molecular oxygen contributes one atom of oxygen to the carbon-19-derived formic acid.[5] Furthermore, the oxygen originally attached to C-3 of the steroid is retained throughout the reaction, suggesting that Schiff base formation at C-3 is not required in the third step.[8]

Despite these and other findings, the chemical nature of the third step remains elusive. Several theories have been shown to be unlikely including 2-hydroxylation,[9] 4,5-epoxidation,[10] Baeyer–Villiger-type oxygen insertion,[5] 10β-hydroxy formation,[11] and any mechanism involving water incorporation into formic acid. An early proposal that has remained consistent with all known aromatase data and P-450 mechanistic theory was made by Akhtar.[12] It features nucleophilic attack of the heme ferric peroxide intermediate on the aldehyde group of **3** as shown in

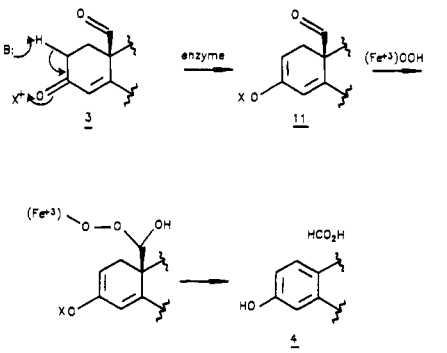

Figure 3.

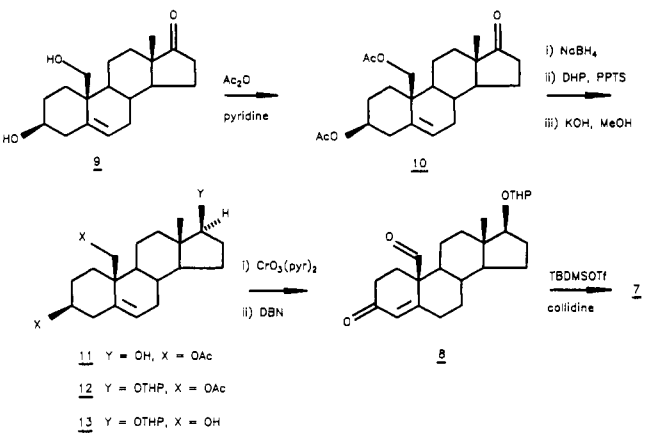

Figure 4.

Figure 2. The resultant hydroxyferric peroxide species **5** could then decompose by hydride shift,[12] proton transfer,[13] or single electron pathways[14] to the aromatic steroid and formic acid. The attractive aspect of this theory is that it accounts for the molecular oxygen incorporation into formate. Furthermore, the proposed six-membered transition state in the peroxide fragmentation reaction would be consistent with the stereoselective loss of the 1β-hydrogen.

Given the limitations of present technology, such a theory is still difficult to rigorously validate. We have recently studied synthetic 19-peroxide analogues of the proposed Akhtar intermediate under a variety of different chemical conditions.[15] While these analogues reacted in some unusual and interesting ways, in no case did they form estrogens (or estrogen derivatives).

We speculated that if the enzyme reaction's third step proceeded according to Akhtar's ferric peroxide theory, one way in which the enzyme could encourage aromatization would be to facilitate enolization of the 3-ketone prior to, or concomitant with, peroxide attack on the aldehyde group (see Figure 3). This could influence decomposition of the peroxide in two ways. First, this would prevent an internal Michael addition of the distal peroxide oxygen to the conjugated 4-en-3-one system as observed in the model studies with the hydroperoxide system. Second, prior enolization could favor 1-hydrogen removal and carbon–carbon bond cleavage if the transition state for the reaction were somewhat product-like. In this way, the energy stabilization associated with aromaticity could directly influence the fragmentation process.

To address this question, we chose to study the reactivity of 19-peroxy 2,4-dienol 3-ethers.[16] Because of its synthetic accessibility and presumed relative stability, a *tert*-butyldimethylsilyl

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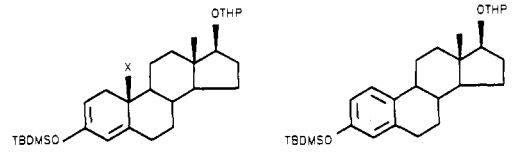
2,4-dienol 3-ether derivative was an attractive target. Previous work on the reaction of hydrogen peroxide with 19-oxo-androstenedione (**3b**) indicated transient formation of the 19-hydroxy 19-hydroperoxide species. We hoped that hydrogen peroxide and *tert*-butyldimethylsilyl enol ether 19-oxo compound would form (at least in equilibrium) the corresponding 19-hydroxy 19-hydroperoxide adduct.

Results and Discussion

After establishing that hydrogen peroxide showed no significant reaction with 10β-methyl dienol ether **6**[17] after several days at 4 °C, we embarked on the synthesis of the corresponding 19-oxo dienol ether **7**. The known[18] 17β-*O*-(tetrahydropyranyl)-19-



6 X = CH_3

7 X = CHO

14

oxotestosterone (**8**) was prepared in a straightforward manner from 19-hydroxy-dehydroepiandrosterone (**9**) as shown in Figure 4. Selective kinetic enolization of the enone **8** toward C-2 to afford the homoannular silyloxydiene **7** was accomplished with *tert*-butyldimethylsilyl triflate and collidine.[19] A solution of the air-sensitive *tert*-butyldimethylsilyl enol ether **7** in CH_2Cl_2–MeOH could be stored at −20 °C in the presence of $NaHCO_3$(anhyd) for several days, without detectable decomposition.

In the event, reaction of compound **7** in 0.88 M HOOH solution in the presence of $NaHCO_3$ (anhyd) afforded the estrogen derivative **14** in 62% yield, after 3 days at 4 °C. The structural assignment of compound **14** was confirmed by chromatographic and spectroscopic comparison with authentic material, which was prepared from estrone (**4b**).[16] Under similar conditions, *tert*-butyl hydroperoxide also converted the aldehyde **7** into the estrogen derivative **14**, although at a somewhat reduced rate [7:3 ratio of **7** to **14** after 3 days on the basis of the product's NMR spectrum]. In contrast, less than 1% conversion took place in the absence of peroxide.

On the basis of ^1H NMR analysis, the hydrogen peroxide reaction produces approximately 1 equiv of formic acid, which has been shown not to be derived from hydrolysis of methyl formate. The formic acid was fully characterized as the *p*-bromophenacyl formate derivative.[20] The lack of reactivity of the 10β-methyl dienol ether **6**, the stoichiometric formation of formic acid, and the stability of the silyloxy group throughout the aromatization, support an aromatization process involving the intermediacy of the 19-hydroxy 19-hydroperoxide **15**. We have thus called this new reaction an aromatase model reaction.

To examine the fidelity of this model reaction to the actual enzymatic reaction, it was necessary to establish the origin of the formic acid oxygen atoms. Reaction of 50%-^{18}O-labeled hydrogen peroxide with the dienol ether **7** led to the formation of formic acid, which for convenience, was derivatized as *p*-bromophenacyl formate. Mass spectral ^{18}O analysis of the *p*-bromophenacyl formate produced in this way indicated that it contained 50.2% atoms of ^{18}O. Furthermore, the fragmentation pattern of the mass spectrum showed that the ^{18}O was indeed present in the formate moiety, because the M − $C_2H_3O_2$ fragment [(bromophenyl)-acylium] showed no isotopic enrichment. On the basis of the initial ^{18}O level in the hydrogen peroxide, there was therefore a stoichiometric incorporation of one oxygen atom from hydrogen peroxide per molecule of formic acid produced. In this respect, the model reaction is faithful to the aromatase reaction.

In order to learn more about the mechanism of the model reaction, we examined the effects of strong base (KOH). It is known that nucleophilic hydrogen peroxide reactions are often drastically accelerated by strong base. To assess the reactivity of the *tert*-butyldimethylsilyl dienol ether with strong base alone, aldehyde **7** was exposed to KOH in the absence of peroxide at 4 °C in a CH_2Cl_2–MeOH solution. There was a slow but steady formation of 17β-*O*-(tetrahydropyranyl)-19-nortestosterone[21] (60% yield after 20 h). We surmised that this was the result of KOH-induced desilylation[17] and reketonization, and that the resultant conjugated ketone **8** then underwent deformylation[22] under these conditions.

Upon treatment with an equimolar ratio of hydrogen peroxide and KOH at 4 °C, compound **7** initially suffered conversion to the aromatic product **14** (TLC), which was relatively rapid in comparison to the standard model reaction which lacked KOH. The amount of aromatic product **14** did not significantly increase with time, but instead a more polar component, which was later identified as 17β-*O*-(tetrahydropyranyl)estradiol,[23] developed. After 4 h, workup of the reaction resulted in the isolation of 17β-*O*-(tetrahydropyranyl)estradiol (40%) in addition to the aromatic product **14** (15%). We inferred that compound **14** was readily undergoing desilylation under the reaction conditions.

That compound **8** was not giving rise to 17β-*O*-(tetrahydropyranyl)estradiol under these conditions was demonstrated in a separate experiment. Furthermore, Mastalerz and Morand have demonstrated that 19-oxoandrostane-3,17-dione 4β,5β-epoxide, another potential side product under these conditions, did not undergo aromatization in the presence of KOH.[10b] Moreover, it was shown that compound **14** was desilylated under identical conditions (KOH, HOOH; 97% yield after 8 h), at a rate consistent with the above hypothesis.

We thus assume that the basic pathway of peroxide attack on the aldehyde group, followed by fragmentation, is still followed in the KOH-catalyzed model reaction. The rate acceleration cannot be quantified precisely, because the presence of KOH causes solubility problems, and because the lifetime of HOOH is known to be shorter in strongly basic solution.[24] Nevertheless, we can make the conservative estimate that there is at least a 10-fold rate enhancement in the presence of KOH.

As mentioned earlier, the 1β-hydrogen of the androgen substrate is lost to the aqueous medium in the enzymatic process. A faithful model should show similar stereoselectivity. We thus synthesized the 1α-deuterio substrate **23**. The synthesis involved several modifications of our previous synthesis of compound **7** (see Figure 5). The diacetate **12** underwent selective methanolysis in the presence of potassium carbonate, giving alcohol **16** in 73% yield. Oxidation of the 3-hydroxy group with Collins reagent to yield **17**, followed by DBN-catalyzed double bond isomerization furnished the known[18] conjugated ketone **18** in 77% overall yield. It should be noted that the Collins/DBN procedure has been found to be a useful alternative for several cases when the Oppenauer oxidation for conversion of 5-en-3β-ol systems to 4-en-3-ones was ineffective. Dehydrogenation of enone **18** using benzeneseleninic anhydride according to the method of Barton[25] [in the presence of $NaHCO_3$(anhyd) to prevent THP removal] smoothly generated the 1,4-dien-3-one **19** in 82% yield. Regio- and stereoselective reduction of the 1,2-double bond with deuterium gas, using Wilkinson's catalyst as described by Djerassi[26] for a similar system, gave the deuteriated enone **20** in 77% yield. Treatment with KOH–MeOH cleaved the 19-acetate and simultaneously led to the desired deuterium exchange from C-2 into solvent.[27a] The

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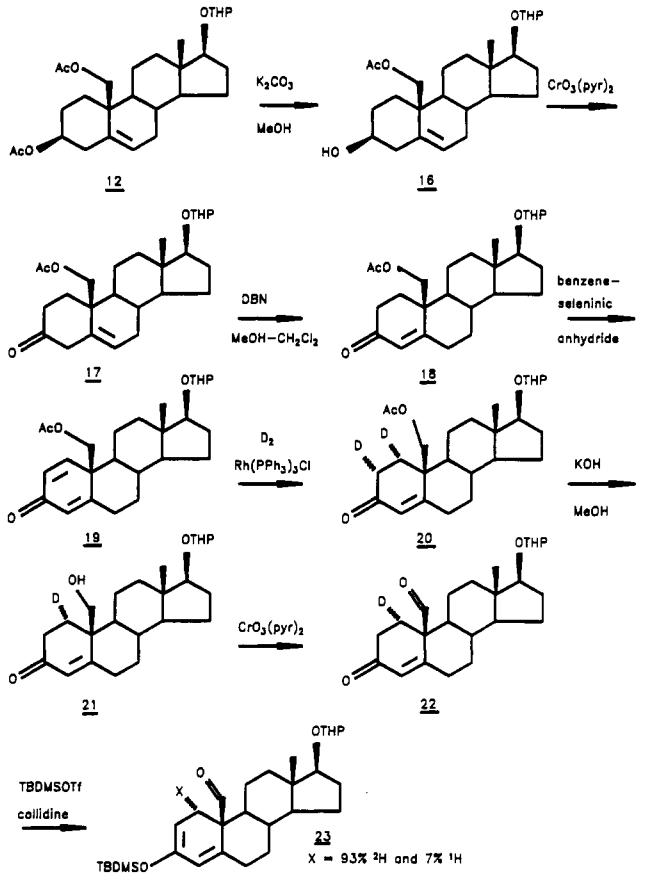

Figure 5.

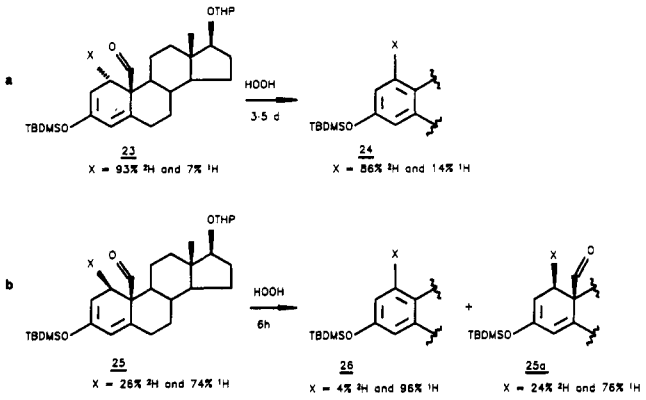

Figure 6. (a) Aromatization of 1α-deuterium-labeled dienol ether **23**. (b) Aromatization of 1β-deuterium-labeled dienol ether **25**.

1α-deuterium-labeled 19-ol **21** was thus produced in 63% yield. Oxidation of **21** with Collins reagent to the labeled aldehyde **22** proceeded quantitatively. Regiospecific enolization of **22**, as described previously, generated the labeled substrate **23**. NMR analysis[27b] of **23** indicated that there was 93% 1α-deuterium incorporation. Furthermore, the weak 1α-proton signal (ca. 7%) that was present in the NMR spectrum of **23** appeared as a symmetric doublet. This suggests that wherever there is a 1α-H, there is also a 1β-H. Reaction of **23** with hydrogen peroxide under the standard model conditions occurred with no detectable change in rate, relative to unlabeled substrate, and led to very high deuterium retention (86% according to NMR analysis) in the aromatic product **24** (see Figure 6a). These findings suggest that the aromatization process was highly stereoselective (approximately 13:1 1α-hydrogen versus 1β-hydrogen). An intrinsic kinetic isotope effect that would not be rate-limiting for the overall reaction could not be ruled out, however.

In order to verify the stereoselectivity of hydrogen loss, the epimeric 1β-deuterio dienol ether analogue **25** was required. This was obtained by selective dehydrogenation of deuteriated enone **20**, followed by stereospecific reduction with H_2. A variety of methods were tested for the dehydrogenation, and the best case (the Saegusa procedure)[28] resulted in incorporation of about 26% 1β-deuterium[27b] at C-1 of the dienol ether **25** with no detectable 1α-deuterium.[29]

We submitted this material to the standard aromatization conditions, but interrupted the reaction after 6 h (21% conversion according to the NMR spectrum). We thus isolated both the starting material (as **25a**) and the aromatic product **26** and obtained ^{1}H NMR spectra for each (see Figure 6b). No significant change in the deuterium content in the starting material was seen (given the estimated experimental error ±2% in deuterium quantitation). An intrinsic kinetic isotope effect of greater than 2 according to the equation of Bigeleisen and Wolfsberg was therefore unlikely.[30] Furthermore, the product showed substantial deuterium loss at C-1 (4% deuterium located at C-1). This suggested that the aromatization was highly stereoselective, and that a possible kinetic isotope effect could play a small role at most in producing the results in the 1α-deuterio case. Because of the larger error associated with the relatively low deuterium enrichment in the 1β-D studies, we cannot rule out small isotope effects (<2).

It was also of interest to compare the kinetics of the model reaction to the known enzymatic reaction. We thus required a convenient assay to define more precisely the rate at 37 °C, and an HPLC assay was developed. As expected, the kinetics were consistent with pseudo-first-order formation of aromatic product

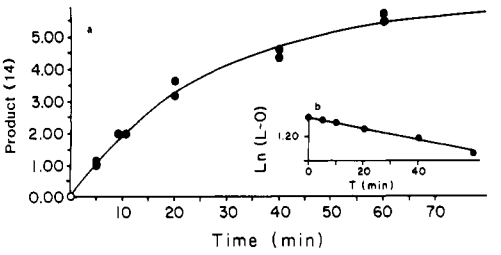

Figure 7. Aromatase model reaction kinetics at 37 °C and 0.88 M HOOH: (a) nonlinear curve fit of estrogen derivative **14** formation (HPLC peak area, arbitrary units) vs time, (b) semilog plot of L minus O (arbitrary units) vs time (L = calculated limiting amount of product **14** at infinite time, O = observed amount of **14** at the specified time.

Table I.[a] Aromatase Model Reaction at 37 °C

conditions	k^b (min^{-1})	product **14** yield[c] (%)
0.22 M HOOH	0.001	64
0.44 M HOOH	0.020	67
0.88 M HOOH	0.039	64
1.67 mM EDTA (0.88 M HOOH)	0.045	64
9.0 mM BHT (0.88 M HOOH)	0.048	80

[a] Based on at least 12 points per assay; initial concentration of substrate [7] = 0.34 mM; experimental errors in rate constants are estimated to be ±15% reported value; errors in product yields are estimated to be ±3% reported yield; see Figure 7 and Experimental Section for details. [b] Pseudo-first-order rate constant. [c] Limiting value.

(27) (a) See Cole, P. A. Ph.D. Thesis, Johns Hopkins University, 1990, for details of hydrogen exchange at C-2. (b) Experimental errors associated with NMR deuterium analysis were estimated to be ±2–3%. (c) ^{1}H NMR assignment of the C-1 protons of **7** is based on the splitting patterns, chemical shifts, standard decoupling experiments, and deuterium-labeling experiments. Thus, **22** a precursor to **23**, showed nearly complete 1α-deuterium incorporation on the basis of the rigorous assignments reported previously.[7a]

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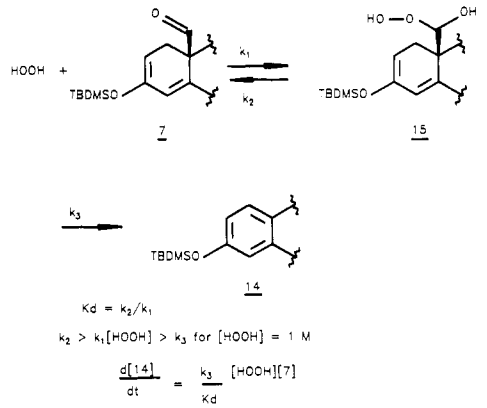

Figure 8.

(initial conditions, 0.88 M HOOH, 0.3 mM *tert*-butyldimethylsilyl dienol ether; see Figure 7). The best fit pseudo-first-order rate constant k was 0.039 min^{-1}, and the calculated limiting product value was equivalent to about 64% yield. This is similar to the isolated yield that was observed for the larger scale 4 °C reactions. The reaction was shown to be approximately first-order with respect to HOOH concentration below 0.88 M HOOH (see Table I). The second-order rate constant was thus found to be 0.0439 M^{-1} min^{-1}.

The metal ion sequestering agent EDTA and the radical scavenger BHT each had only minor effects on the reaction rate. BHT led to a significantly increased limiting product value (the equivalent of 80% yield), suggesting that aromatization in the model reaction does not require initiation by radicals, and that long-lived radical intermediates are not involved. Indeed, the increased yields in the presence of BHT imply that BHT actually inhibits side reactions. Rate studies with larger amounts of peroxide were problematic due to solubility difficulties. Interestingly, there was a small but reproducible increase in rate as a function of water concentration in the reaction (data not shown), but the details and origin of this effect have not been fully explored.

A postulated kinetic description of the reaction is shown in Figure 8. It is thought that there is a rapid equilibrium between the aldehyde **7** and the hydroxy hydroperoxide **15**. Even at 0.88 M HOOH, the equilibrium appears to lie heavily toward the left, on the basis of NMR studies with the 19-oxoandrost-5-ene-3β,17β-diol and HOOH,[15b] and the fact that the reaction is cleanly first-order in hydrogen peroxide. This implies that K_d is substantially greater than 0.88 M. A relatively slow step (k_3) from unstable intermediate **15** to the aromatic product **14** is postulated, because the HOOH epoxidation of compound **3b** is considerably faster than the aromatization reaction at the same temperature.[15b] This difference probably arises from discrepancies in the rates of breakdown toward product of the respective intermediates, rather than major differences in the initial equilibrium step. Furthermore, attack of HOOH on aldehydes is generally considered to be quite fast.[31] The most likely effect of the potassium hydroxide is to increase k_1 relative to k_2 and thereby bias the equilibrium toward **15**. Additionally, the strong base might play a role in increasing k_3 if a proton-transfer step is partially rate-determining.

The current evidence disfavors a radical decomposition mechanism for conversion of **15** to **14**, although short-lived radical intermediates cannot be ruled out. Of the hydride-shift or proton-transfer mechanisms, the latter seems chemically more plausible, but this remains to be proved. The interesting stereoselectivity that was observed may be evidence for a six-membered (chair) transition state in the aromatization step (see Figure 9), as was originally proposed by Akhtar for the enzymatic reaction.

It is amusing, if not entirely valid, to compare enzymatic rates to model reaction rates. For purified, reconstituted enzyme, the

(31) Hiatt, R. In *Organic Peroxides*; Swern, D., Ed.; Wiley-Interscience, New York, 1971; Vol. II, Chapter 1.

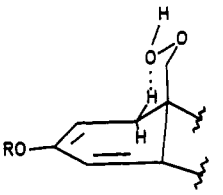

Figure 9.

turnover number for the conversion of aldehyde **3** to estrogen **4** was shown[2c] to be about 20 min^{-1}. A direct comparison of the model reaction rate at 0.88 M HOOH versus this turnover number shows the model reaction is about 500 times slower than the enzyme reaction. Assuming the model reaction would still be first-order in hydrogen peroxide at such a concentration, an effective concentration (E_m) of 450 M HOOH would be necessary to achieve such a rate increase. Of course such a peroxide concentration could not be obtained in our model reaction, but it is conceivable in the active site of an enzyme. Furthermore, the importance of the heme in effecting peroxide cleavage is not known, and it too could lead to rate increases.

In summary, we have developed and explored the mechanism of a reaction that possesses many of the key features of Akhtar's theory for the third step of aromatase. Recent work examining the substrate-dependent stereoselectivity of 2-hydrogen removal in the aromatase reaction has been interpreted as evidence for enzymic assistance in directing 2-hydrogen loss.[7a] On the other hand, it must be admitted that the absence of exchange of 2-deuterium from 2-deuterio-19-oxoandrogens in nonturnover conditions[7a] (that is, aromatase in the absence of NADPH) provides no clear evidence supporting an active-site base.

While further work is necessary to test the relevance of this model reaction to the actual enzymatic conversion, it provides chemical plausibility to previously uncharted terrain. Furthermore, the recent finding[32] that 3-desoxy-2,4-dien-19-oxoandrogen is a potent aromatase competitive inhibitor and alternate substrate lends credence to these model studies as representative of the actual enzymatic reaction.

Experimental Section

General Procedure. IR spectra were recorded on a Perkin-Elmer 710B instrument. UV spectra were obtained on a Perkin-Elmer Lambda 3B machine. ^1H NMR spectra were measured in $CDCl_3$ solution and referenced to $CHCl_3$ (7.26 ppm) by using an IBM FT-80 spectrometer or a Varian XL-400 spectrometer. Flash chromatography was performed according to the method of Still, using Baker flash silica gel. HPLC was performed on a Beckman instrument with UV detection, using a reverse-phase analytical column (Altex ultrasphere octyl). TLC was performed by using fluorescence indicator Macherey-Nagel D-5160 plates. 19-Hydroxydehydroepiandrosterone was obtained as a gift from Syntex Co. All compounds and solvents were commercially available reagent grade unless otherwise noted. Mass spectra were recorded on a VG70S instrument. The ^{18}O-labeled hydrogen peroxide was obtained from ICON, Summit, NJ.

3β,19-*O*,*O*'-Diacetyl-19-hydroxydehydroepiandrosterone (10). To a stirred solution of diol **9** (2.4 g, 7.89 mmol) in pyridine (21 mL, 20.5 g, 259 mmol) under N_2 at room temperature was added 4-(dimethylamino)pyridine (600 mg, 4.91 mmol) and acetic anhydride (5.6 mL, 6.06 g, 59.4 mmol). After 105 min, the mixture was transferred to a separatory funnel containing H_2O (200 mL) and CH_2Cl_2 (200 mL). The separated organic phase was washed two times with 5% aqueous HCl (50 mL each) followed by saturated aqueous $CuSO_4$ (150 mL), and the organic phase was dried ($MgSO_4$) and concentrated in vacuo to afford the known[33] diacetate **10** (2.9 g, 7.47 mmol, 95% yield) as a white solid: ^1H NMR (80 MHz) δ 5.69 (m, 1 H, 6-H), 4.9–4.45 (br m, 1 H, 3-H), 4.26 (ABq, J_{AB} = 12 Hz, $\Delta\nu_{AB}$ = 48.6 Hz, 2 H, 19-CH_2), 2.04 (s, 3 H, CH_3CO_2R), 2.02 (s, 3 H, CH_3CO_2R), 0.90 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 2960, 1740 cm^{-1}; CIMS (M + NH_4^+) m/z 406, calcd for (M − HOAc) $C_{21}H_{28}O_3$ 328.2039, found 328.2038.

(32) Cole, P. A.; Bean, J. M.; Robinson, C. H. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2999.

(33) Sunthankar, S. V.; Vakatkar, V. V. *J. Chem. Soc., Chem. Commun.* **1976**, 913.

3β,19-*O*,*O*'-Diacetylandrost-5-ene-3β,17β,19-triol (11). To a stirred solution of ketone **10** (100 mg, 0.258 mmol) in 4:1 ethanol–dioxane (10 mL) under N_2 at room temperature was added $NaBH_4$ (15 mg, 0.40 mmol). After 1.5 h, the resultant mixture was partitioned between CH_2Cl_2 (70 mL) and H_2O (40 mL). The aqueous phase was further extracted with CH_2Cl_2 (30 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated in vacuo to afford alcohol **11** (97 mg, 0.25 mmol, 96% yield): ^{1}H NMR (80 MHz) δ 5.65 (m, 1 H, 6-H), 4.65 (br m, 1 H, 3-H), 4.22 (ABq, J_{AB} = 11.9 Hz, $\Delta\nu_{AB}$ = 44.5 Hz, 2 H, 19-CH_2), 3.68 (m, 1 H, 17-H), 2.05 (s, 3 H, CH_3CO$_2$R), 2.02 (s, 3 H, CH_3CO$_2$R), 0.77 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 3550, 2910, 1720 cm^{-1}; CIMS (M + NH_4^+) *m/z* 408, calcd for (M − HOAc) $C_{21}H_{30}O_3$ 330.2195, found 330.2199.

3β,19-*O*,*O*'-Diacetyl-17β-*O*''-(tetrahydropyranyl)androst-5-ene-3β,17β,19-triol (12). To a stirred solution of the alcohol **11** (60 mg, 0.15 mmol) in THF (5 mL) under N_2 at room temperature was added dihydropyran (0.25 mL, 231 mg, 2.75 mmol) and pyridinium *p*-toluenesulfonate (40 mg, 0.16 mmol). After the disappearance of starting material (14 h, TLC), the reaction mixture was partitioned between CH_2Cl_2 (60 mL) and H_2O (60 mL). The aqueous phase was further extracted with CH_2Cl_2 (30 mL), and the combined organic phases were washed with brine, dried (Na_2SO_4), and concentrated in vacuo. The crude residue was flash chromatographed (12 g of SiO_2, 20% EtOAc–hexanes) to afford pure tetrahydropyranyl ether **12** (72 mg, 0.152 mmol, 99% yield): ^{1}H NMR (80 MHz) δ 5.64 (m, 1 H, 6-H), 4.70 (br m, 1 H, 3-H), 4.61 (br s, 1 H, ROC*H*OR'), 4.23 (ABq, J_{AB} = 11.9 Hz, $\Delta\nu_{AB}$ = 43 Hz, 2 H, 19-CH_2), 4.0–3.3 (m, 3 H), 2.03 (s, 3 H, CH_3CO$_2$R), 2.01 (s, 3 H, CH_3CO$_2$R), 0.80 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 2930, 1720 cm^{-1}; CIMS (M + NH_4^+) *m/z* 492, calcd for (M − HOAc) $C_{26}H_{38}O_4$ 414.2770, found 414.2777.

17β-*O*-(Tetrahydropyranyl)androst-5-ene-3β,17β,19-triol (13). The diacetate **12** (50 mg, 0.105 mmol) was dissolved in a stirred solution of 7.5% KOH–MeOH (10 mL) under N_2 at room temperature. After 9.5 h, the reaction mixture was transferred to a separatory funnel and partitioned between CH_2Cl_2 (80 mL) and H_2O (40 mL). The organic phase was further washed with H_2O (30 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo to afford diol **13** (38 mg, 0.097 mmol, 93% yield) as a pure white solid. ^{1}H NMR (80 MHz) δ 5.75 (m, 1 H, 6-H), 4.63 (br s, 1 H, ROC*H*OR'), 4.0–3.3 (m, 6 H), 0.85 (s, 1.5 H, 18-CH_3), 0.84 (s, 1.5 H, 18-CH_3); IR ($CHCl_3$) 3600, 2920 cm^{-1}; CIMS (M + NH_4^+) *m/z* 408, calcd for (M − H_2O) $C_{24}H_{36}O_3$ 372.2664, found 372.2674.

17β-*O*-(Tetrahydropyranyl)-19-oxotestosterone (8). To a stirred solution of diol **13** (200 mg, 0.513 mmol) in dry CH_2Cl_2 (30 mL) under N_2 at room temperature was added a slurry of Collins reagent (1.6 g, 6.2 mmol) in CH_2Cl_2 (10 mL) over a period of 1 min. The resultant mixture was allowed to stir until the starting material had disappeared (30 min, TLC). The mixture was transferred to a florisil column (10 g) packed in CH_2Cl_2. The column was eluted with CH_2Cl_2 (100 mL) followed by EtOAc (300 mL), and the total eluate was combined and concentrated in vacuo. The crude residue was dissolved in 1:1 CH_2Cl_2–MeOH (80 mL), and 1,5-diazabicyclo[4.3.0]non-5-ene (0.7 mL, 704 mg, 5.7 mmol) was added. The solution was allowed to stand at room temperature until there was complete conversion of starting material to a more polar UV-active component (20 min, TLC). The mixture was partitioned between sodium phosphate buffer (0.1 M, pH 7) (300 mL) and CH_2Cl_2 (300 mL). The aqueous phase was further extracted with CH_2Cl_2 (100 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. The resultant was flash chromatographed (25 g of silica, 25% EtOAc–hexanes) to afford the known[18] compound **8** (89 mg, 0.23 mmol, 45% overall yield) as a colorless oil: ^{1}H NMR (80 MHz) δ 9.95 (s, 1 H, 19-H), 5.97 (s, 1 H, 4-H), 4.61 (s, 1 H, ROC*H*OR'), 4–3.3 (m, 3 H), 0.79 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 2850, 1670, 1610 cm^{-1}; UV (MeOH) λ_{max} 245 nm (ε 10000); CIMS *m/z* calcd for (M) $C_{24}H_{34}O_4$ 386.2457, found 386.2461.

3-*O*-(*tert*-Butyldimethylsilyl)-17β-*O*'-(tetrahydropyranyl)-19-oxoandrosta-2,4-diene-3,17β-diol (7). To a stirred solution of enone **8** (15 mg, 0.0389 mmol) in dry benzene (1 mL) under N_2 was added freshly distilled collidine (0.17 mL, 156 mg, 1.29 mmol), and the solution was cooled to 0 °C. The mixture was treated with *tert*-butyldimethylsilyl triflate (0.08 mL, 92 mg, 0.35 mmol) and allowed to stir for 40 min. The mixture was transferred to a separatory funnel containing saturated aqueous $NaHCO_3$ (25 mL) and CH_2Cl_2 (40 mL). After partitioning, the separated aqueous phase was further extracted with CH_2Cl_2 (10 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. The crude product was flash chromatographed (0.5 g of SiO_2, 5% EtOAc–hexanes containing 1 drop of Et_3N/10 mL), affording homoannular dienol ether **7** (18 mg, 0.036 mmol, 93% yield) as a pure (HPLC, TLC) air-sensitive white solid. Compound **7** was stored in CH_2Cl_2–MeOH solution, in the presence of saturating anhydrous $NaHCO_3$, at −20 °C where it was stable for days: HPLC (reverse phase, ultrasphere octyl analytical column, eluent 100% MeOH, flow rate 0.7 mL/min, retention time 8 min); ^{1}H NMR (400 MHz) δ 9.72 (s, 0.5 H, 19-H), 9.70 (s, 0.5 H, 19-H), 5.62 (s, 1 H, 4-H), 4.72 (d, *J* = 6 Hz, 1 H, 2-H), 4.60 (br s, 1 H, ROC*H*OR'), 3.85 (m, 1 H), 3.61 (q, *J* = 9 Hz, 1 H), 3.45 (m, 1 H), 3.0 (m, 1 H, 1β-H),[27c] 2.50 (br t, *J* = 14.2 Hz, 1 H, 6β-H), 2.39 (br d, *J* = 14.2 Hz, 1 H, 6α-H), 2.19 (br d, *J* = 17.4 Hz, 1α-H), 0.89 (s, 9 H, *tert*-butyl), 0.78 (s, 1.5 H, 18-CH_3), 0.77 (s, 1.5 H, 18-CH_3), 0.09 (s, 3 H, CH_3Si), 0.08 (s, 3 H, CH_3Si); IR ($CHCl_3$) 2820, 1710, 1650 cm^{-1}; UV (hexane) λ_{max} 282 nm (ε 3300); CIMS *m/z* calcd for (M) $C_{30}H_{48}O_4Si$ 500.3322, found 500.3326.

3-*O*-(*tert*-Butyldimethylsilyl)-17β-*O*'-(tetrahydropyranyl)estradiol (14). To a solution of dienol ether **7** (5.5 mg, 0.011 mmol) in 20:1 MeOH–CH_2Cl_2 (1.05 mL) containing anhydrous $NaHCO_3$ (ca. 3 mg) was added aqueous 30% HOOH (0.1 mL, 30 mg, 0.88 mmol), and the vortexed reaction mixture was allowed to stand at 4 °C for 72 h. The resultant mixture was transferred to a separatory funnel containing aqueous 3% sodium thiosulfate (40 mL) and CH_2Cl_2 (50 mL). After partitioning, the separated aqueous phase was further extracted with CH_2Cl_2 (15 mL) and the combined organic phases were dried ($MgSO_4$) and concentrated in vacuo. Flash chromatography (0.5 g of SiO_2, 4% EtOAc–hexanes) of the crude residue afforded the estrogen derivative **14** (3.2 mg, 0.0068 mmol, 62% yield): ^{1}H NMR (400 MHz) δ 7.12 (d, *J* = 8 Hz, 0.5 H, 1-H), 7.10 (d, *J* = 8 Hz, 0.5 H, 1-H), 6.60 (br d, *J* = 8 Hz, 1 H, 2-H), 6.54 (br s, 1 H, 4-H), 4.67 (m, 1 H, ROC*H*OR'), 3.92 (m, 1 H), 3.72 (m, 1 H), 3.49 (m, 1 H), 2.79 (m, 2 H, 6-CH_2), 0.98 (s, 9 H, *tert*-butyl), 0.83 (s, 1.5 H, 18-CH_3), 0.81 (s, 1.5 H, 18-CH_3), 0.19 (s, 6 H, (CH_3)$_2$Si); IR ($CHCl_3$) 2900, 1600 cm^{-1}; UV (hexane) λ_{max} 276 nm (ε 1600); CIMS *m/z* calcd for (M) $C_{29}H_{46}O_3Si$ 470.3216, found 470.3220.

Reaction of Dienol Ether 7 with *t*-BuOOH. To a solution of the dienol ether **7** (5.0 mg, 0.01 mmol) in 20:1 MeOH–CH_2Cl_2 (1.05 mL) containing anhydrous $NaHCO_3$ (ca. 3 mg) was added aqueous 70% *t*-BuOOH (0.1 mL, 70 mg, 0.78 mmol). The mixture was vortexed and allowed to stand for 72 h at 4 °C. The mixture was concentrated under a stream of N_2 and then in vacuo. The ^{1}H NMR (400 MHz) spectrum of the crude reaction mixture indicated that the ratio of **7** to **14** was 7:3.

Reaction of Dienol Ether 7 with HOOH. To a solution of the dienol ether **7** (10 mg, 0.02 mmol) in 20:1 MeOH–CH_2Cl_2 (2 mL) containing anhydrous $NaHCO_3$ (6 mg) was added aqueous 30% HOOH (0.1 mL, 30 mg, 0.88 mmol). The mixture was vortexed and allowed to stand for 72 h at 4 °C. The mixture was concentrated under a stream of N_2 and then in vacuo. The ^{1}H NMR (400 MHz) spectrum of the crude reaction mixture indicated that the ratio of **7** to **14** was 1:25.

Reaction of Dienol Ether 7 without Peroxides. A solution of dienol ether **7** (5 mg, 0.01 mmol) in 20:1 MeOH–CH_2Cl_2 (1.05 mL) containing anhydrous $NaHCO_3$ (ca. 3 mg) was allowed to stand for 72 h at 4 °C. The mixture was concentrated under a stream of N_2 and then in vacuo. The ^{1}H NMR (400 MHz) spectrum indicated that the ratio of **7** to **14** was greater than 99:1.

Formic Acid Quantitation in the Transformation of Dienol Ether 7 to Estrogen Derivative 14. The aromatization of **7** with HOOH was performed two more times (reactions 1 and 2) as previously described. The workup involved adding anhydrous Na_2CO_3 (5 mg) to the crude reaction mixtures and concentrating the reaction mixtures under a jet of N_2. The white solid residues were dissolved in D_2O (1 mL) containing *n*-propanol (1 mg) as standard, and ^{1}H NMR spectra were obtained. Comparison of the integrals of the propanol CH_2OH with the formate hydrogen after corrections for a standard mixture allowed quantification of the formate yield. The aqueous insoluble residues were flash chromatographed as previously described, and the products **14** were quantitated on the basis of weight. The reactions 1 and 2 gave **14** yields of 50% and 56%, respectively, and formate yields of 50% and 72%, respectively. The overall average was therefore 1.1 mol of formate/mol of **14** isolated.

***p*-Bromophenacyl Formate Derivatization of the Formic Acid from the Reaction of Dienol Ether 7 with HOOH.** The aromatization of dienol ether **7** (30 mg, 0.06 mmol) was performed as described above. The crude reaction mixture was treated with anhydrous Na_2CO_3 (ca. 3 mg) before concentration to dryness under a stream of N_2. The white solid residue was dissolved in D_2O (0.5 mL), and a ^{1}H NMR spectrum indicated that significant sodium formate was present (comparison to a spectrum of an authentic sample). After the D_2O sample was diluted with several mL of H_2O, the solution was carefully neutralized to pH 7.5 with aqueous 10% HCl. This solution was concentrated in vacuo and dissolved in EtOH (2 mL), and then *p*-bromophenacyl bromide (31 mg, 0.11 mmol) was added. The mixture was warmed to 90 °C in an oil bath for 60 min under N_2. The reaction mixture was cooled to room temperature and concentrated under a stream of N_2 and then in vacuo (20 mmHg) for several hours. The crude residue was flash chromatographed (5 g of SiO_2, 20% EtOAc–hexanes) to afford pure *p*-bromophenacyl formate (4 mg, 0.016 mmol, 27% yield based on **7**). The formate de-

rivative prepared in this way showed TLC, IR, UV, ^{1}H NMR, and MS data identical with those of the known[20] compound.

17β-O-(Tetrahydropyranyl)-19-nortestosterone. To a solution of dienol ether **7** (7.0 mg, 0.014 mmol) in CH_2Cl_2 (0.5 mL) was added 5% KOH–MeOH solution (1 mL, 50 mg, 0.89 mmol), and the vortexed solution was allowed to stand at 4 °C. After 2 h, the starting material appeared to be partially converted to a more polar compound (TLC). After 20 h, the mixture was partitioned between aqueous 0.1 M sodium phosphate buffer (50 mL, pH 7) and CH_2Cl_2 (50 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (30 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. The crude residue was flash chromatographed (0.5 g of SiO_2, 18% EtOAc–hexanes) to afford 17β-O-(tetrahydropyranyl)-19-nortestosterone (3.0 mg, 0.0084 mmol, 60% yield), which was identical with authentic material[21] on the basis of comparison of the TLC, IR, UV, ^{1}H NMR, and MS data.

17β-O-(Tetrahydropyranyl)estradiol. A solution of the dienol ether **7** (7 mg, 0.014 mmol) in CH_2Cl_2 (0.7 mL) and 5% KOH–MeOH (1 mL, 50 mg, 0.89 mmol) was treated with aqueous 30% HOOH (0.1 mL, 30 mg, 0.88 mmol), and the vortexed, heterogeneous mixture was left standing at 4 °C. After 15 min, significant conversion to **14** was observed (TLC). Monitoring of the reaction (TLC) over the next 3 h showed that the amount of **14** stayed relatively constant while **7** decreased and a more polar component (17β-O-(tetrahydropyranyl)estradiol) increased. After 4 h, the starting material had nearly completely disappeared (TLC) and the mixture was partitioned between aqueous saturated $NaHCO_3$ (50 mL) and CH_2Cl_2 (40 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (40 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. The crude product was flash chromatographed (0.5 g of SiO_2, 17% EtOAc–hexanes) to afford **14** (1.0 mg, 0.0021 mmol, 15% yield) and 17β-O-(tetrahydropyranyl)estradiol[23] (2 mg, 0.0056 mmol, 40% yield). The spectral data for 17β-O-(tetrahydropyranyl)estradiol were ^{1}H NMR (400 MHz) δ 7.16 (d, J = 8 Hz, 0.5 H, 1-H), 7.14 (d, J = 8 Hz, 0.5 H, 1-H), 6.63 (br d, J = 8 Hz, 0.5 H, 2-H), 6.61 (br d, J = 8 Hz, 0.5 H, 2-H), 6.54 (br s, 1 H, 4-H), 4.68 (m, 1 H, ROC*H*OR'), 3.92 (m, 1 H), 3.72 (m, 1 H), 3.50 (m, 1 H), 2.81 (m, 2 H, 6-CH_2), 0.82 (s, 1.5 H, 18-CH_3), 0.80 (s, 1.5 H, 18-CH_3); IR ($CHCl_3$) 3600, 2900, 1600 cm^{-1}; UV (MeOH) λ_{max} 279 nm (ε 1600); CIMS m/z calcd for (M) $C_{23}H_{32}O_3$ 356.2351, found 356.2354.

Reaction of 17β-O-(Tetrahydropyranyl)-19-oxotestosterone (8) with KOH–HOOH. To a solution of the enone **8** (10 mg, 0.028 mmol) in CH_2Cl_2 (0.1 mL) and 5% KOH–MeOH (1 mL, 50 mg, 0.89 mmol) was added aqueous 30% HOOH (0.1 mL, 30 mg, 0.88 mmol), and the heterogeneous mixture was vortexed and left to stand for 4 h at 4 °C. The crude mixture was partitioned between aqueous saturated $NaHCO_3$ (40 mL) and CH_2Cl_2 (40 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (40 mL). The combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. The ^{1}H NMR spectrum (400 MHz) of the crude product indicated that less than 5% formation of 17β-O-(tetrahydropyranyl)estradiol had occurred.

19-O-Acetyl-17β-O'-(tetrahydropyranyl)androst-5-ene-3β,17β,19-triol (16). To a stirred solution of the diacetate **12** (24 mg, 0.051 mmol) in MeOH (3 mL) was added anhydrous K_2CO_3 (8.0 mg, 0.058 mmol) under N_2 at room temperature. After 4.5 h, the mixture was partitioned between aqueous dilute NH_4Cl (40 mL) and CH_2Cl_2 (30 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (30 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. Flash chromatography (3 g of SiO_2, 33% EtOAc–hexanes) of the crude product afforded monoacetate **16** (16 mg, 0.037 mmol, 73% yield): ^{1}H NMR (80 MHz) δ 5.6 (m, 1 H, 6-H), 4.61 (br s, 1 H, ROC*H*OR'), 4.22 (ABq, J_{AB} = 11.8 Hz, $\Delta\nu_{AB}$ = 38.3 Hz, 2 H, 19-CH_2), 4.0–3.3 (m, 4 H), 2.04 (s, 3 H, CH_3CO$_2$R), 0.79 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 3600, 2920, 1720 cm^{-1}; CIMS (M + H$^+$) m/z 433, calcd for (M − H_2O) $C_{26}H_{40}O_5$ 414.2770, found 414.2775.

19-O-Acetyl-17β-O'-(tetrahydropyranyl)androst-5-ene-17β,19-diol-3-one (17). To a vigorously stirred solution of the alcohol **16** (100 mg, 0.231 mmol) in CH_2Cl_2 (5 mL) was added Collins reagent (320 mg, 1.24 mmol) under N_2 at room temperature. After 30 min, the reaction appeared complete (TLC), and the reaction mixture was passed through a florisil column (10 g, packed in CH_2Cl_2), eluting with CH_2Cl_2 (100 mL) followed by EtOAc (300 mL). The combined eluates were concentrated in vacuo and flash chromatographed (12 g of SiO_2, 35% EtOAC–hexanes) to afford the ketone **17** (88 mg, 0.205 mmol, 89% yield) as a colorless oil: ^{1}H NMR (400 MHz) δ 5.59 (m, 1 H, 6-H), 4.63 (m, 1 H, ROC*H*OR'), 4.35 (ABq, J_{AB} = 12.1 Hz, $\Delta\nu_{AB}$ = 186.6 Hz, 1 H, 19-CH_2), 4.35 (ABq, J_{AB} = 11.8 Hz, $\Delta\nu_{AB}$ = 180.1 Hz, 1 H, 19-CH_2), 3.89 (m, 1 H), 3.65 (m, 1 H), 3.49 (m, 1 H), 3.36 (br d, J = 16.9 Hz, 1 H, 4β-H), 2.87 (d, J = 16.9 Hz, 1 H, 4α-H), 2.17 (s, 1.5 H, CH_3CO$_2$R), 2.07 (s, 1.5 H, CH_3CO$_2$R), 0.84 (s, 1.5 H, 18-CH_3), 0.83 (s, 1.5 H, 18-CH_3); IR ($CHCl_3$) 2920, 1730, 1710 cm^{-1}; CIMS m/z calcd for (M) $C_{26}H_{38}O_5$ 430.2719, found m/z 430.2713.

19-O-Acetyl-17β-O'-(tetrahydropyranyl)androst-4-ene-17β,19-diol-3-one (18). To a stirred solution of the unconjugated ketone **17** (2.2 g, 5.12 mmol) in 5:1 CH_2Cl_2–MeOH (120 mL) was added 1,5-diazabicyclo-[4.3.0]non-5-ene (0.875 mL, 0.88 g, 7.09 mmol) under N_2 at room temperature. After 4 h, the reaction appeared complete (TLC), and the mixture was partitioned between aqueous 0.1 M sodium phosphate buffer (500 mL, pH 7) and CH_2Cl_2 (300 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (300 mL). The combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. The crude residue was purified by flash chromatography (150 g of SiO_2, 35% EtOAc–hexanes) to afford the known conjugated ketone **18** (1.90 g, 4.42 mmol, 86% yield) as a colorless oil: ^{1}H NMR (80 MHz) δ 5.90 (s, 1 H, 4-H), 4.60 (br s, 1 H, ROC*H*OR'), 4.42 (ABq, J_{AB} = 11.3 Hz, $\Delta\nu_{AB}$ = 39.5 Hz, 2 H, 19-CH_2), 4.0–3.3 (m, 3 H), 1.99 (s, 3 H, CH_3CO$_2$R), 0.81 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 2940, 1730, 1660, 1615 cm^{-1}; UV (MeOH) λ_{max} 238 nm (ε 16 000); CIMS m/z calcd for (M) $C_{26}H_{38}O_5$ 430.2719, found 430.2717.

19-O-Acetyl-17β-O'-(tetrahydropyranyl)androsta-1,4-diene-17β,19-diol-3-one (19). To a stirred solution of the enone **18** (400 mg, 0.930 mmol) in chlorobenzene (20 mL) containing anhydrous $NaHCO_3$ (400 mg, 4.76 mmol) was added benzeneseleninic anhydride (500 mg, 1.39 mmol) under N_2, and the mixture was warmed to 100 °C. After 6 h, the reaction mixture was cooled to room temperature and partitioned between aqueous 0.1 M sodium phosphate buffer (70 mL, pH 7.1) and CH_2Cl_2 (40 mL). The aqueous phase was further extracted with CH_2Cl_2 (40 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. The residue was flash chromatographed (50 g of SiO_2, 24% EtOAc–hexanes) to afford pure dienone **19** (325 mg, 0.759 mmol, 82% yield): ^{1}H NMR (80 MHz) δ 7.08 (d, J = 10.1 Hz, 1 H, 1-H), 6.29 (dd, J = 10.1, 1.8 Hz, 1 H, 2-H), 6.18 (br s, 1 H, 4-H), 4.57 (br s, 1 H, ROC*H*OR'), 4.53 (ABq, J_{AB} = 10.6 Hz, $\Delta\nu_{AB}$ = 13.8 Hz, 2 H, 19-CH_2), 4.0–3.3 (m, 3 H), 1.88 (s, 3 H, CH_3CO$_2$R), 0.82 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 2900, 1740, 1660, 1620, 1600 cm^{-1}; UV (MeOH) λ_{max} 241.5 nm (ε 14 700); CIMS m/z calcd for (M − THP) $C_{21}H_{28}O_4$ 344.1988, found 344.1990.

Transformation of 1,4-Diene Compound 19 to 4-Ene Compound 18. A vigorously stirred solution of the dienone **19** (30 mg, 0.0701 mmol) in 1:1 benzene–MeOH (15 mL) was degassed with aspirator vacuum and regassed with H_2 delivered from a balloon. The cycle was repeated three times over 15 min. To the solution was added $Rh(PPh_3)_3Cl$ (28 mg, 0.0303 mmol) and another degas/regas cycle was performed. Within 2 min, the color of the solution turned from red to yellow. The solution gradually became darker again, and after 3 h the reaction appeared nearly complete (TLC). The mixture was filtered through florisil (10 g, packed in CH_2Cl_2) eluting with CH_2Cl_2 (25 mL) followed by EtOAc (50 mL). The combined eluates were flash chromatographed (10 g of SiO_2, 23% EtOAc–hexanes) to afford pure monoenone **18** (20 mg, 0.0465 mmol, 66% yield) as a colorless oil. Compound **18** had TLC, ^{1}H NMR, and IR data identical with those of the known compound.

[1α,2α-$^2H_2$]-19-O-Acetyl-17β-O'-(tetrahydropyranyl)androst-4-ene-17β,19-diol-3-one (20). The dienone **19** (55 mg, 0.129 mmol) was reduced with D_2 analogously as described in the unlabeled case [1:1 benzene–MeOH (6 mL), $Rh(PPh_3)_3Cl$ (30 mg, 0.0324 mmol), D_2 gas]. The crude reaction mixture was concentrated in vacuo and flash chromatographed (10 g of SiO_2, 23% EtOAc–hexanes) to afford labeled monoenone **20** (43 mg, 0.0995 mmol, 77% yield). Compound **20** had identical R_f (TLC) and very similar ^{1}H NMR data compared to the unlabeled compound **18**. Mass spectroscopic analysis for deuterium in **20** (M − THP$^+$) indicated d_0, 3%; d_1, 27%; d_2 69%.

[1α-^{2}H]-17β-O-(Tetrahydropyranyl)androst-4-ene-17β,19-diol-3-one (21). The labeled enone **20** (30 mg, 0.0694 mmol) was dissolved in 5% KOH–MeOH (20 mL) and allowed to stand at room temperature under N_2 for 11 h. The pale yellow solution was partitioned between CH_2Cl_2 and 50 mM sodium phosphate buffer (pH 7.1) and worked up in standard fashion. The crude material was flash chromatographed (8 g of SiO_2, 50% EtOAc–hexanes) to furnish the labeled 19-alcohol **21** (17 mg, 0.0437 mmol, 63% yield) as a pure white solid. Compound **21** had identical TLC and very similar ^{1}H NMR data compared to the unlabeled compound.[34] Mass spectroscopic analysis for deuterium in **21** (M − THP$^+$) indicated d_0, 7%; d_1, 92%; d_2, 1%.

[1α-^{2}H]-17β-O-(Tetrahydropyranyl)-19-oxotestosterone (22). To a vigorously stirred solution of the labeled 19-alcohol **21** (12.5 mg, 0.0321 mmol) in CH_2Cl_2 (100 mL) was added Collins reagent (100 mg, 0.388 mmol) under N_2 at room temperature. After 5 min, the reaction appeared complete (TLC) and the reaction was worked up and purified similarly to the method described for the synthesis of **8** to afford labeled

(34) Cole, P. A. Ph.D. Thesis, Johns Hopkins University, 1990.

19-aldehyde **22** (12 mg, 0.0310 mmol, 97% yield). Compound **22** had identical TLC and very similar ^{1}H NMR data compared to those of the unlabeled compound **8**. Mass spectroscopic analysis for deuterium in **22** ($M - THP^+$) revealed that d_0, 8.6%; d_1, 89.3%; d_2, 2.1%.

[1α-^{2}H]-3-*O*-(*tert*-Butyldimethylsilyl)-17β-*O*'-(tetrahydropyranyl)-19-oxoandrosta-2,4-diene-3,17β-diol (23). The labeled enone **22** (10 mg, 0.0258 mmol) in benzene (0.8 mL) was reacted with collidine and TBDMS triflate as described previously. Workup and purification were performed analogously to the reaction with unlabeled material to afford pure labeled dienol ether **23** (9.0 mg, 0.018 mmol, 70% yield). Compound **23** had identical TLC properties and very similar ^{1}H NMR data compared to those of the unlabeled compound **7**. From the 400-MHz ^{1}H NMR spectrum of **23**, it was determined that compound **23** contained 93% 1α-^{2}H with no detectable 1β-^{2}H (1β-H appeared as a broad singlet, residual 1α-H appeared as a symmetrical doublet). Mass spectroscopic analysis (M^+) for deuterium indicated d_0, 8.6%; d_1, 90.0%; d_2, 1.1%.

Transformation of [1α-^{2}H]-3-*O*-(*tert*-Butyldimethylsilyl)-17β-*O*'-(tetrahydropyranyl)-19-oxoandrosta-2,4-diene-3,17β-diol (23) into [1-^{2}H]-3-*O*-(*tert*-Butyldimethylsilyl)-17β-*O*'-(tetrahydropyranyl)estradiol (24). To a solution of labeled dienol ether **23** (7.5 mg, 0.015 mmol) in 20:1 MeOH-CH_2Cl_2 (1.05 mL) containing anhydrous $NaHCO_3$ (ca. 3 mg) was added aqueous 30% HOOH (0.1 mL, 30 mg, 0.88 mmol). The vortexed reaction mixture was allowed to stand at 4 °C for 3.5 days, after which time workup and purification were performed as for the reaction with the unlabeled material. The labeled estrogen derivative **24** (4.5 mg, 0.00955 mmol, 64% yield) was furnished as a white solid. The TLC properties of compound **24** were identical with those of the unlabeled estrogen derivative **14**. The ^{1}H NMR (400 MHz) spectrum of **24** was similar to that of **14** except that the 1-H signal was greatly reduced (*d*, 86%) and the 2-H signal appeared largely as a broad singlet (*d*, 0%). Mass spectroscopic analysis of **24** (M^+) for deuterium indicated d_0, 18.3%; d_1, 81.4%; d_2, 0.3%.

Transformation of Labeled Dienol Ether 25 to 25a and Estrogen Derivative 26. The labeled dienol ether **25**[29] (4.0 mg, 0.00798 mmol; 26% deuterium at the 1β-position, less than 3% deuterium in the 1α-position) was reacted with HOOH as previously described for the unlabeled compound **7**, except that, after 6 h, the reaction was quenched with aqueous saturated $Na_2S_2O_3$ (5 mL). The resultant mixture was partitioned between aqueous half-saturated $NaHCO_3$ (40 mL) and CH_2Cl_2 (40 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (20 mL), and the combined organic phases were dried (Na_2SO_4) and concentrated in vacuo. One-fourth of the crude residue was set aside for a ^{1}H NMR spectrum, which indicated that the ratio of dienol ether **25a** to labeled estrogen derivative **26** was 79:21. The remaining three-fourths of the crude material was purified by flash chromatography (0.5 g of SiO_2, 4% EtOAc-hexanes containing 1 drop of Et_3N/10 mL) to afford **25a** (ca. 1.5 mg) and **26** (ca. 0.5 mg). ^{1}H NMR analysis of compound **25a** indicated that there was about 24% deuterium in the 1β-position with negligible deuterium in the 1α-position. ^{1}H NMR analysis of compound **26** indicated that there was about 4% deuterium at the 1-position.

Model Reaction Kinetic Studies. General Procedure. Test tubes containing 3 mg of anhydrous $NaHCO_3$ were charged with 1:50 CH_2Cl_2-MeOH (0.9 mL) solution containing dienol ether **7** (171 μg, 0.342 μmol) and placed in a Dubnoff shaker bath at 37 °C for ca. 2 min. The reactions were initiated with 0.1 mL of aqueous HOOH solution (2.2, 4.4, or 8.8 M HOOH). After vortexing for several seconds, the reactions were incubated for varying time lengths, e.g., 0, 5, 10, 20, 40, and 60 min for 0.88 M HOOH assays. Assays run with EDTA contained 10 μL of an aqueous 167 mM EDTA solution (pH 7.2). Assays run with BHT contained 2 mg (9 μmol) of BHT. Assay reactions were quenched with aqueous saturated sodium thiosulfate (4 mL), and then immediately partitioned between aqueous one-third saturated NH_4Cl (60 mL) and CH_2Cl_2 (20 mL). The separated aqueous phase was further extracted with CH_2Cl_2 (20 mL), and the combined organic phases were dried (Na_2SO_4), concentrated in vacuo, analyzed for product **14** levels with reverse-phase HPLC (Altex ultrasphere octyl analytical column, eluent 100% MeOH, flow rate 0.7 mL/min, UV detection at 280 nm, retention time 10 min). The zero time point levels showed in all cases less than 1% product **14** formation. All assays were performed in duplicate, and the results were averaged. The data was analyzed by using the Enz-Fit first-order nonlinear regression program, and replots of ln $(P_\infty - P_0)$ versus time gave straight lines with no systematic deviation observed. Errors in the rate constants were estimated to be ±15%.

[^{18}O]-*p*-Bromophenacyl Formate. The dienol ether **7** (14.0 mg, 0.028 mmol) was reacted with randomly labeled aqueous 20% [50%-^{18}O]-HOOH solution analogously to the reaction with unlabeled hydrogen peroxide, and the sodium formate product was converted to the labeled bromophenacyl formate derivative (1.5 mg, 0.0061 mmol, 22% yield) as previously described. The TLC and ^{1}H NMR data of the labeled derivative were identical with those of the unlabeled compound. Mass spectroscopic analysis of the labeled formate derivative for ^{18}O indicated (M) $^{18}O_0$, 49.8%; $^{18}O_1$, 50.2%. The fragment ($M - C_2H_3O_2$) possessed no detectable ^{18}O.

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Probing the Acceptor Specificity of β-1,4-Galactosyltransferase for the Development of Enzymatic Synthesis of Novel Oligosaccharides

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Abstract: β-1,4-Galactosyltransferase has been investigated with regard to its acceptor specificity and used in the synthesis of galactosides with 5-thioglucose, glucal, deoxynojirimycin, modified *N*-acetylglucosamine, and glucose derivatives as acceptors. The galactoside products are potentially useful as endoglycosidase or glycosyltransferase inhibitors or as intermediates for the synthesis of complex oligosaccharides. The conformation of each enzyme product has been investigated with NMR; all are shown to possess similar glycosidic torsional angles based on a significant NOE between H-1 of Gal and H-4 of the acceptor. Comparison of the transferase reactions with the β-1,4-galactosidase-catalyzed galactosyl transfer reactions indicates that the transferase reactions provide exclusively a β-1,4-glycosidic linkage while the galactosidase reactions predominantly form a β-1,6-glycosidic linkage.

The stereocontrolled synthesis of oligosaccharides based on sophisticated protection/deprotection, activation, and coupling strategies has been well established.[2,3] Enzymatic oligosaccharide synthesis based on glycosyltransferases[4] is a useful alternative to

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