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The steric course of enzymic hydroxylation at primary carbon atoms

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1. Introduction

Contrary to earlier ideas (e.g. ref. 1) about the composition of the Earth's primordial atmosphere, it is now generally held that this atmosphere consisted principally of a relatively unreactive mixture of CO, N_2, and H_2O, with only traces of H_2 and reduced sulphur gases.[2,3] Ample evidence documenting the existence of life under these anaerobic conditions is available.[4,5] The first cellular organisms seem to have appeared approximately 3.4 billion years ago.[6,7] Currently, it is considered likely that life originated at the deep sea hydrothermal vents containing abundant amounts of hydrogen sulfides, methyl thiol CH_3SH, carbon monoxide, carbon dioxide, and dark smoke consisting of metal sulfides.[8] Mixed iron–nickel sulfides were shown to catalyse the conversion[8] of methyl thiol and carbon monoxide to methyl thioacetate $CH_3CO(SCH_3)$ having a newly generated C–C bond and resembling acetyl coenzyme A, a known biosynthetic precursor of many biomolecules. The anaerobic organisms were necessarily

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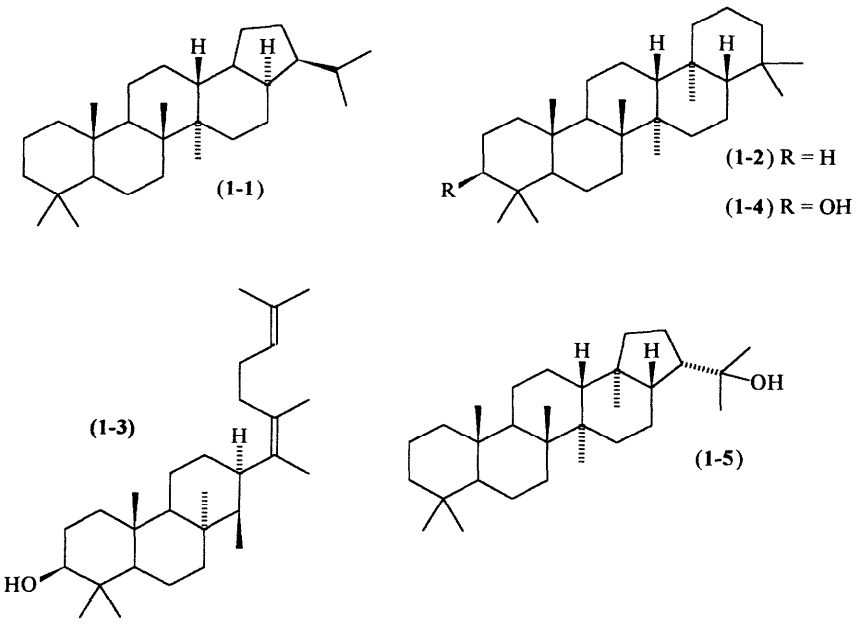
aquatic, since the archaic oceans appear to have afforded conditions uniquely conducive to the evolution of living matter[8] and offered protection against harmful solar ultraviolet radiation in the absence of a shielding ozone layer.[6] These anaerobic organisms are likely to have used both acyclic and cyclic polyprenoids as structural components of their membranes,[9] thereby attesting to the ancient origin of polyprenoids.

The elaboration of cyclic and polycyclic polyprenoids under anaerobic conditions can be rationalized in terms of a cationic enzyme-mediated attack on the terminal double bond of an



Scheme 1.

acyclic precursor.[10] 3-Deoxytriterpenes such as hopane[11] (Scheme 1, Structure **1-1**) or gammacerane (**1-2**) are thought to arise from a *proton* attack on a terminal double bond of squalene, in contrast to the *oxidative* cyclization of squalene which requires molecular oxygen[12,13] to generate (2,3*S*)-epoxysqualene. Enzyme-mediated cationic attack on the oxirane ring of (2,3*S*)-epoxysqualene cleaves the C(2)–O bond, generating an electron deficiency at C-2 and initiating cyclization to 3-hydroxytriterpenes. Depending on the organism, oxidative cyclization of squalene can lead to different hydroxytriterpenes: lanosterol in mammals such as rats,[14] cycloartenol in plants,[14] protosterol (**1-3**) in the fungus *Fusidium coccineum*,[15] etc.

The non-oxidative cyclization of squalene, dating from Earth's anaerobic period, has survived in certain ferns,[16–19] mosses,[20] lichens,[21] fungi,[22] protozoa,[23,24] and bacteria.[25–28] The ciliate *Tetrahymena pyriformis* has an ancient mechanism for biosynthesis of the triterpenes tetrahymanol (**1-4**) and diplopterol (**1-5**),[24] which are then used in the construction of cell membranes. This ancient mechanism is suppressed by exogenous sterols, which are metabolized (e.g. dehydrogenated) and taken up into the membrane. In this manner, exogenous cholesterol is converted to 3β-hydroxycholesta-5,7,22-triene and incorporated into the membrane of *T.*

pyriformis.[23,24] In the absence of steroidal substrates *T. pyriformis* activates its dormant biosynthetic processes and produces polyprenoids according to the sequence:

$$\text{acetate} \rightarrow \text{mevalonic acid (MVA)} \rightarrow \text{squalene} \rightarrow \text{triterpenes}$$

The biosynthesis of tetrahymanol (**1-3**) proceeds anaerobically,[29] the overall process being equivalent to acquisition of the elements of water by squalene.[24,30,31] In certain organisms non-oxidative and oxidative squalene cyclization mechanisms coexist, as evidenced by the isolation of both tetrahymanol and C-3 oxygenated phytosterols from the fern *Oleandra wallichii*.[17,19,32]

Blue–green algae, which appeared some 3.4 billion years ago, introduced photosynthetic production of dioxygen and initiated a slow phasing out of the anaerobic atmosphere.[6,33] Substantial quantities of O_2 probably did not begin to accumulate before the widespread establishment of cyanophytic photosynthesis some 1.8–2.0 billion years ago, approaching its present atmospheric proportion of approximately 20% only about a half-billion years ago.[4] When the atmospheric oxygen achieved a concentration of approximately 10% of its current level a layer of ozone started to form, which provided protection from the devastating ultraviolet radiation. Under its shield, aquatic organisms continued to evolve and eventually emerged from the seas to populate the land.[34] Establishment of an oxidizing terrestrial atmosphere was accompanied by a transition from an exclusively anaerobic to a predominantly aerobic metabolism, with concurrent development of organelles designed to take advantage of the low-potential electron sink which molecular oxygen affords living cells and tissues.[35]

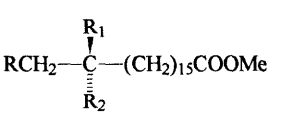
2. Oxidative metabolism

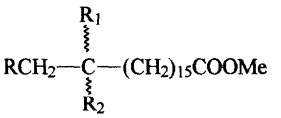
The pivotal role of oxygen in anabolic and catabolic processes is the subject of a massive literature which will not be reviewed here. Suffice it to say that attachment of only one of the two oxygen atoms of molecular oxygen to an unactivated carbon atom to yield an hydroxylated product is an important enzymic process:

$$RH + O_2 + 2H \rightarrow ROH + H_2O$$

That the oxygen atom of the hydroxyl moiety derives from molecular oxygen is evidenced by the incorporation of isotopic oxygen[36] when enzymic incubations are performed in an $^{18}O_2$ atmosphere but not in a $[^{18}O]H_2O$-enriched medium. Mixed-function oxidases can also catalyze other types of reactions, including hydroxylations at heteroatoms, dehalogenations, dealkylations, deaminations, denitrosations, epoxidations, and lactonizations.[37–45]

In the context of studies on the mechanism(s) of reactions catalyzed by mixed-function oxidases, the stereochemistry of the incorporated hydroxyl moiety has been investigated. In this report we summarize work performed in our laboratory on the steric mode of hydroxylation at primary carbon atoms. To facilitate an understanding of the subject we briefly review the stereochemistry of enzymic hydroxylation at unactivated secondary and tertiary carbon atoms. No attempt is made to exhaustively cover the literature, and only selected examples immediately related to the topics under discussion are cited.





(2-1) $R_1 = {}^3H$, $R = R_2 = H$

(2-2) $R = R_1 = H$, $R_2 = {}^3H$

(2-3) $R = R_1 = H$, $R_2 = {}^3H$

(2-4) $R = OH$, $R_1 = H$, $R_2 = {}^3$

(2-5) $R = R_2 = H$, $R_1 = OH$

(2-6) $R = H$, $R_1 = OH$, $R_2 = {}^3H$

Scheme 2.

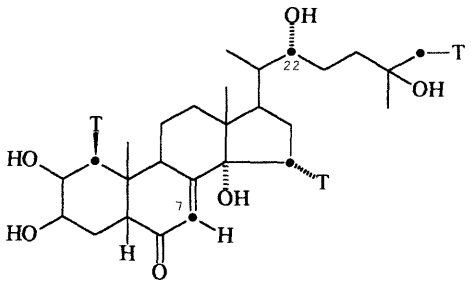
3. Stereochemistry of enzymic hydroxylation at secondary carbon atoms

The steric mode of microbial hydroxylation of long chain aliphatic compounds was investigated using methyl (17*R*)[17-^{3}H]stearate (**2-1**), methyl (17*S*)[17-^{3}H]stearate (**2-2**), and methyl (17*RS*)[17-^{3}H]stearate (**2-3**).[46,47] Each tritiated compound was admixed with methyl [U-^{14}C]stearate and incubated with the yeast *Torulopsis gropengiesseri*.[48] Workup of the incubation mixtures yielded methyl 18-hydroxystearates (**2-4**) and methyl (17*R*)-hydroxystearates. In all instances, 18-hydroxylation proceeded without altering the atomic $^3H:{}^{14}C$ ratios (ar), whereas 17-hydroxylation of substrate **2-3** was accompanied by a loss of tritium. This indicated that the ω and ω-1 hydroxylation reactions are independent and likely involve direct substitution of a hydrogen atom by hydroxyl. Oxygenation at C-17 of substrate **2-1** to yield methyl (17*R*)17-hydroxystearate (**2-5**) proceeded with loss of a tritium atom, while the analogous hydroxylation of substrate **2-2** gave methyl (17*R*)[17-^{3}H]17-hydroxystearate (**2-6**). The results indicate that hydroxylation at a secondary aliphatic carbon atom by *T. gropengiesseri* involves displacement of a hydrogen atom in the retention mode,[46,47] i.e. the incoming hydroxyl group assumes the steric orientation of the displaced hydrogen atom.

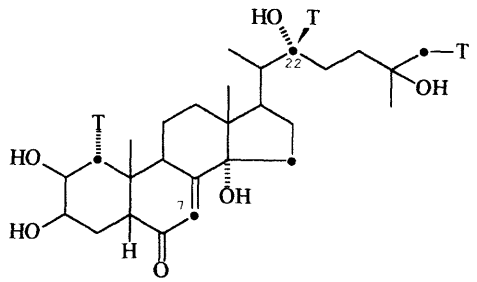
The steric course of hydroxylation at secondary carbon atoms of the aliphatic side chain of sterols by insects was determined using [1,7,15,22,26-$^{14}C_5$;1β,7β,15α,22*R*,26-$^3H_5$]cholesterol ['(*R*)-cholsterol'] biosynthesized by rat liver enzymes from (3*RS*;2*R*)[2-^{14}C;2-^{3}H]MVA and [1,7,15,22,26-$^{14}C_5$;1α,22*S*,26-$^3H_3$]cholesterol ['(*S*)-cholesterol'] biosynthesized by rat liver enzymes from (3*RS*;2*S*)[2-^{14}C;2-^{3}H]MVA.[52] Administration of (*R*)-cholesterol [^{3}H/^{14}C, ar (5:5)] to the locust *Schistocerca gregaria* gave (*R*)-ecdysone (**3-1**) [^{3}H/^{14}C, ar (3:5)] and proceeded with loss of the 7β- and 22*R*-tritium atoms.[53] The complementary experiment with (*S*)-cholesterol [^{3}H/^{14}C, ar (3:5)] produced (*S*)-ecdysone (**3-2**) without loss of tritium. It follows that (22*R*)-hydroxylation of cholesterol in the locust involves abstraction of the 22*R* tritium atom of (*R*)-cholesterol and proceeds with retention of configuration.[53]

The stereochemistry of hydroxylation at secondary carbon atoms of the steroidal nucleus has been a subject of numerous investigations. Incubation of [11α,12α-$^3H_2$]5β-pregnane-3,20-dione (**4-1**) with the fungus *Rhizopus stolonifer* produced [12α-^{3}H]11α-hydroxypregnane-3,20-dione (**4-2**) with loss of the 11α-^{3}H atom.[54] The same organism converted [11β-^{2}H]5β-prenane-3,20-dione (**4-3**) to [11β-^{2}H]11α-hydroxypregnane-3,20-dione (**4-4**) without loss of deuterium.[55] As expected, *R. stolonifer* transformed [9α,11α,12α-$^2H_3$]5β-prenane-3,20-dione (**4-5**) to the

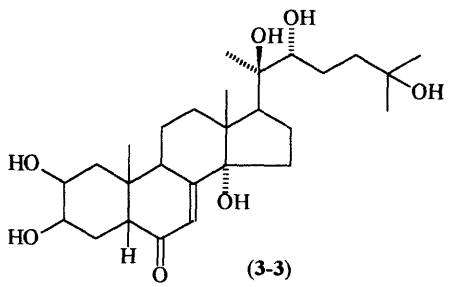
[T = tritium, ^{3}H; • = ^{14}C]



(3-1)

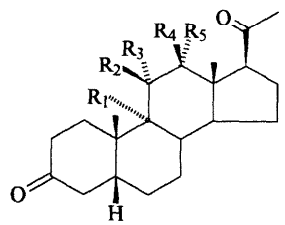


(3-2)



(3-3)

Scheme 3.



(4-1) $R_1 = R_2 = R_4 = H$, $R_3 = R_5 = {}^{3}H$

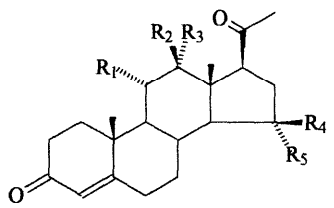
(4-2) $R_1 = R_2 = R_4 = H$, $R_3 = OH$, $R_5 = {}^{3}H$

(4-3) $R_1 = R_3 = R_4 = R_5 = H$, $R_2 = {}^{2}H$

(4-4) $R_1 = R_4 = R_5 = H$, $R_2 = {}^{2}H$, $R_3 = OH$

(4-5) $R_1 = R_3 = R_5 = {}^{2}H$, $R_2 = R_4 = H$

(4-6) $R_1 = R_5 = {}^{2}H$, $R_2 = R_4 = H$, $R_3 = OH$



(4-7) $R_1 = R_3 = {}^{3}H$, $R_2 = R_4 = R_5 = H$

(4-8) $R_1 = R_3 = {}^{3}H$, $R_2 = R_5 = OH$, $R_4 = H$

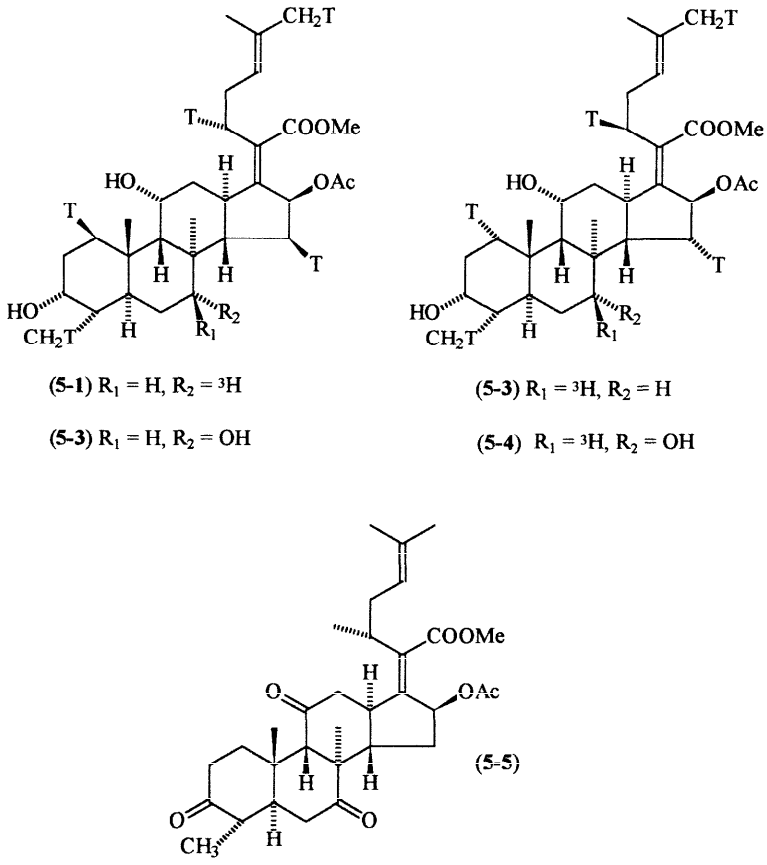
(4-9) $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = {}^{3}H$

(4-10) $R_1 = R_3 = H$, $R_2 = R_5 = OH$, $R_4 = {}^{3}H$

Scheme 4.

11α-hydroxy analogue (**4-6**) with loss[54] of 11α-^{2}H. The fungus *Calonectria decora* metabolized [11α,12α-$^3H_2$]progesterone (**4-7**) to [11α,12α-$^3H_2$]12β,15α-dihydroxyprogesterone (**4-8**) with retention of both tritium atoms.[56] Similarly, incubation of [15β-^{3}H]progesterone (**4-9**) with *C. decora* gave a 12β,15α-diol (**4-10**) which retained the 15β-tritium atom.[57] Incubation of [15β-^{3}H]progesterone (**4-9**) with *Penicillium* spp. ATCC 11598 produced [15β-^{3}H]15α-hydroxyprogesterone (**4-10**) without loss of the isotope.[57]

From the known stereochemistry of incorporation of hydrogen atoms of MVA into squalene[52] and the mode of cyclization of (2,3*S*)-epoxysqualene into protosterol (**1-3**), which in turn is metabolized to fusidic acid,[58] the (*R*)-fusidic acid (**5-1**) produced from [2*R*-^{3}H]MVA, and

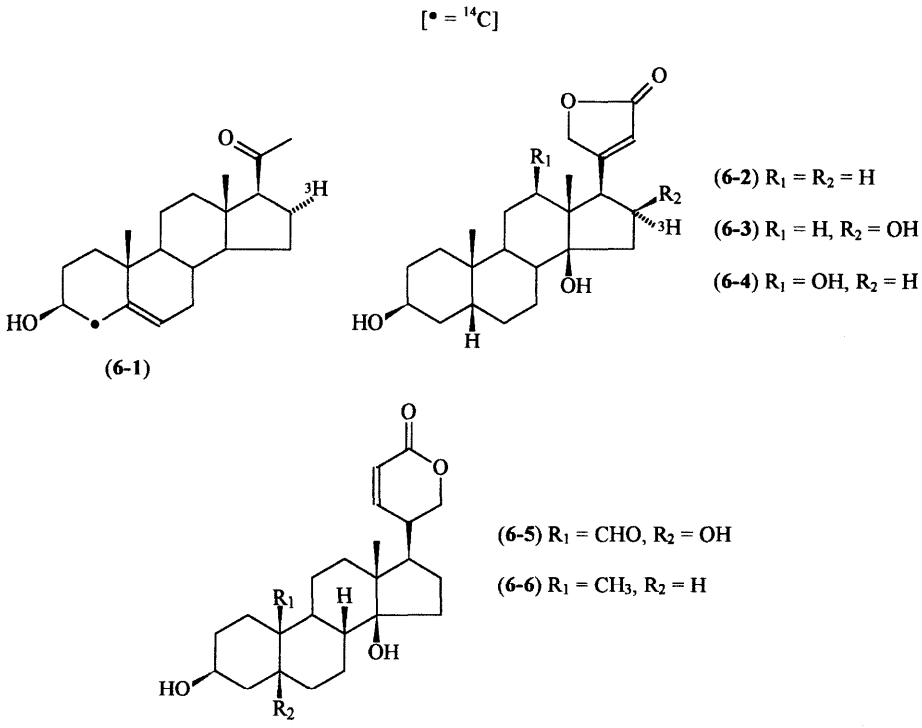


Scheme 5.

the (*S*)-fusidic acid (**5-2**) produced from [2*S*-^{3}H]MVA should contain a 7α-^{3}H atom and a 7β-^{3}H atom, respectively.[59] Location of the isotopes was confirmed by incubating samples of **5-1** and **5-2** with a helvolic acid-producing strain of *Acremonium persicinum*,[60] which metabolizes fusidic acid to its 7α-hydroxy analogue. Conversion of **5-1** to 7α-hydroxyfusidic acid (**5-3**) proceeded with loss of the 7α-^{3}H atom, and no further loss of tritium occurred upon oxidation of **5-3** to the corresponding 3,7,11-trione (**5-5**).[60] In contrast, formation of 7α-hydroxyfusidic acid **5-4** from

5-2 did not entail a loss of tritium, though a tritium atom was lost upon oxidation of **5-4** to the trione (**5-5**).[60]

Hydroxylation at secondary carbon atoms of steroids by plants also proceeds with retention of configuration. Following administration of [4-^{14}C;16α-^{3}H]pregnenolone (**6-1**) [^{3}H/^{14}C,



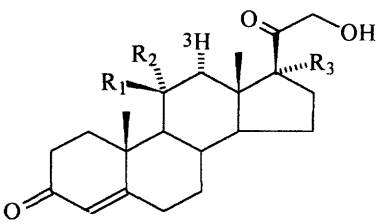
Scheme 6.

ar (1:1)] to *Digitalis purpurea* (the common foxglove), the recovered digitoxigenin (**6-2**) and gitoxigenin (16α-hydroxydigitoxigenin, **6-3**) retained all the tritium present in the administered substrate.[61]

Mammalian hydroxylation at secondary steroidal carbon atoms similarly proceeds stereospecifically in the retention mode. Bovine adrenal perfusion of [11α,12α-$^{3}H_2$]progesterone (**4-7**) resulted in [$^{3}H_2$]cortisol (**7-1**) and [$^{3}H_2$]corticosterone (**7-2**).[54] Insertion of the 11β-hydroxyl occurred without loss of tritium from substrate **4-7**.[54] Transformation of [4-^{14}C;7α-^{3}H]cholesterol in rats yielded cholic acid (**7-3**) devoid of tritium, demonstrating that insertion of the 7α-hydroxyl proceeds with retention of configuration.[62]

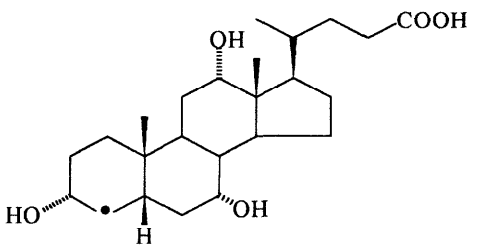
The very large body of evidence, some of which is summarized previously, establishes beyond reasonable doubt that microbial, plant, and mammalian hydroxylations at unactivated secondary carbon atoms proceed stereospecifically in the retention mode. The possibility that 15β-hydroxylation of a hypothetical cholesterol precursor proceeds with inversion of configuration was suggested;[63] however, experimental evidence in support of this hypothesis has not been forthcoming.

[• = ^{14}C]



(7-1) $R_1 = R_3 = OH$, $R_2 = {}^3H$

(7-2) $R_1 = OH$, $R_2 = {}^3H$, $R_3 = H$



(7-3)

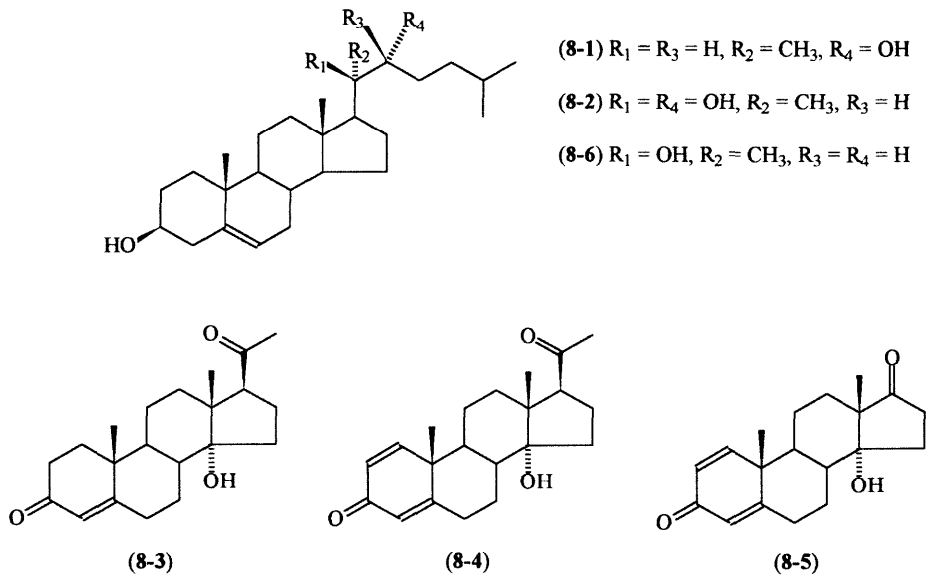
Scheme 7.

4. Stereochemistry of enzymic hydroxylation at tertiary carbon atoms

While hydroxylation reactions at unactivated secondary carbon atoms uniformly proceed in the retention mode, the stereochemistry of enzymic hydroxylations at *tertiary* carbon atoms seems to be species-dependent and may occur with either retention or inversion of configuration. Hydroxylation at C-20 of (22*R*)-22-hydroxycholesterol (**8-1**) by adrenal enzymes takes place in the retention mode to give (20*R*,22*R*)-20,22-dihydroxycholesterol (**8-2**).[64] Similarly, insertion of the C-20 hydroxyl in the elaboration of 20-hydroxyecdysone (**3-3**) from cholesterol also proceeds in the retention mode.[53]

A strain of the fungus *Colletotrichum antirrhini*[65] (SC 2144) metabolized progesterone to several products, including 14α-hydroxyprogesterone (**8-3**), 14α-hydroxy-1-dehydroprogesterone (**8-4**), and 14α-hydroxyandrosta-1,4-diene-3,17-dione (**8-5**).[69] Insertion of the 14α-hydroxy group proceeded with retention of configuration. Likewise, introduction of a 14α-hydroxyl group during biosynthesis of ecdysones **3-1** and **3-2** from cholesterol by locusts proceeds in the retention mode.[53]

Biosynthesis of the cardenolides digitoxigenin (**6-2**), gitoxigenin (**6-3**), and digoxigenin (**6-4**) from pregnenolone, progesterone,[70–75] deoxycorticosterone,[76] cholesterol,[77–79] sitosterol,[80] and (20*S*)-20-hydroxycholesterol (**8-6**)[76] has been documented. Cardenolides **6-2**, **6-3**, and **6-4** each have a 14β-hydroxyl group, while the administered substrates all have the 14α-H stereochemistry. It is apparent that introduction of the 14β-hydroxyl moiety of cardenolides involves an *overall* inversion of configuration at C-14. 14β-Hydroxylation during biosynthesis of the plant bufadienolide hellebrigenin (**6-5**) from progesterone proceeds with inversion of configuration;[71] bufalin (**6-6**) biosynthesized from cholesterol or bile acids by the toad *Bufo marinus* also has a 14β-hydroxy moiety.



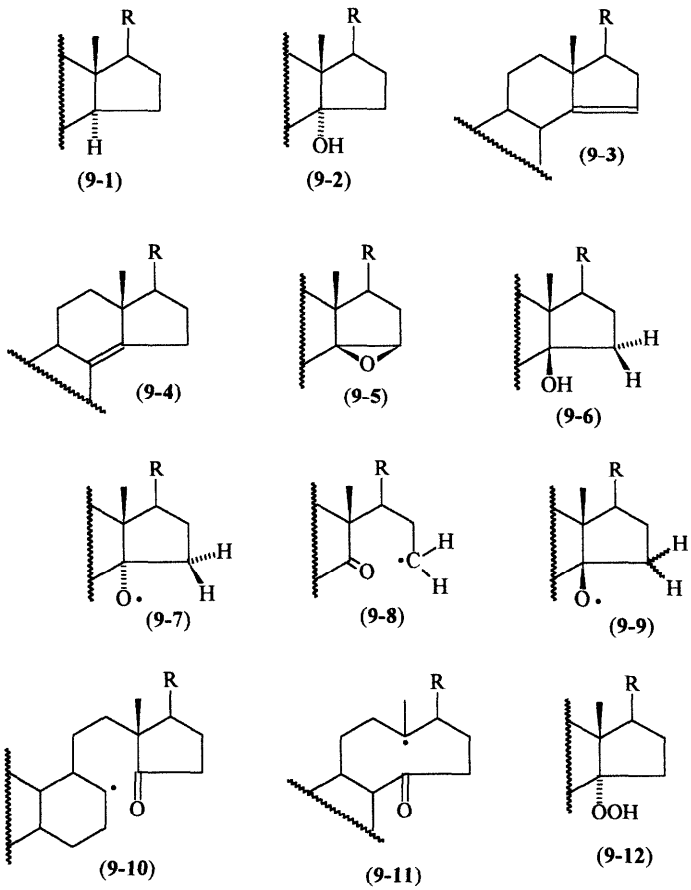
Scheme 8.

The question naturally arises how to rationalize the various stereochemical options for hydroxylations at unactivated tertiary carbon atoms. To simplify matters we will focus on C-14 hydroxylation of steroidal substrates having the 14α-H stereochemistry. As indicated previously, depending on the species, either 14α- or 14β-hydroxylated products are obtained.[78,81]

Hydroxylations at tertiary carbon atoms with *retention* of configuration may be viewed as proceeding in a manner analogous to hydroxylations at secondary carbon atoms. In contrast, several mechanisms can be envisaged for hydroxylation reactions proceeding in the inversion mode.[78,81] The latter reaction may involve a process formally equivalent to a Walden inversion,[82] implying that the eliminated hydrogen atom departs from the α-face, opposite to the trajectory of the incoming hydroxyl group. A simple, straightforward Walden-type hydroxylation at C-14 seems unlikely on mechanistic grounds. The ease with which a carbon center undergoes an S_N2 reaction decreases in the order primary > secondary > tertiary;[83] the steroidal C-14 position, being crowded and rigid, is an unlikely candidate for a classical Walden inversion reaction. Moreover, a classical Walden inversion at the C-14 position implies departure of a hydride ion (H^-) which, unless somehow 'assisted', is a poor leaving group; indeed, there seems to be no precedent for the unassisted nucleophilic displacement of a hydride ion from an unactivated carbon center.[84–86]

We have considered several alternative mechanisms consistent with known biochemical transformations and which are amenable to experimental evaluation. We started with the premise that a cardenolide precursor such as **9-1** could be hydroxylated in the 'normal' manner with retention of configuration to generate a 14α-hydroxy intermediate (**9-2**); dehydration of **9-2** would yield either the 14(15)-olefin (**9-3**) or the 8(14)-olefin (**9-4**). Epoxidation at the β-face of olefin **9-3** gives 14β(15)-epoxide (**9-5**) which, following reductive opening of the oxirane ring, yields the 14β-hydroxy compound **9-6**. A similar sequence of transformations can be postulated

for olefin **9-4**. Note that olefins **9-3** and **9-4** can also be derived via dehydration of the C-15 or C-8 alcohols, respectively.



Scheme 9.

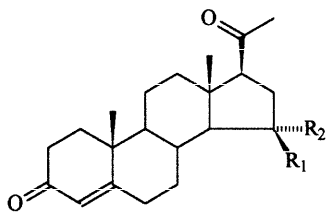
A free radical route,[81] whereby the 14α-hydroxy intermediate **9-2** gives rise to a 14α-oxa radical (**9-7**), was also considered. Rearrangement of **9-7** to a 14-keto-15-radical **9-8** and its subsequent closure on the α-face would give the 14β-oxa radical **9-9** and then the required 14β-hydroxy product **9-6**. Similar rearrangements of keto radicals **9-10** and **9-11** would likewise yield the 14β-hydroxy product **9-6**.

To evaluate the role, if any, of the 8(14)-olefin, [8-^{3}H;4-^{14}C]cholesterol was administered to *Digitalis lanata* (the Grecian foxglove). The recovered digitoxigenin[87,88] and digoxigenin had the same ^{3}H/^{14}C ar as the substrate. These results exclude the intermediacy of an olefin encompassing C-8.[89] Samples of digitoxigenin biosynthesized by *D. lanata* from (i) (2*R*,3*R*)-[2-^{3}H;2-^{14}C]MVA, (ii) [15β-^{3}H]progesterone, and (iii) [15-^{3}H$_2$,21-^{3}H$_2$]progesterone retained the anticipated amounts of tritium at C-15, indicating that a 14(15)-olefinic intermediate does *not* participate in the elaboration of cardenolides by this plant.[89]

To test the hypothetical oxyradical sequence **9-7→9-8→9-6** we administered a mixture of [1-^{3}H]14α-hydroxyprogesterone and [4-^{14}C]progesterone to *D. lanata*. The recovered digitoxigenin contained ^{14}C but was devoid of tritium,[81] suggesting that 14α-hydroxyprogesterone either is not transported to the site of cardenolide biosynthesis, or else is not a precursor of cardenolides in this plant. However, these results do not preclude the intermediacy of other 14α-hydroxy C_{21}-compounds.

Initial formation of 14α-hydroperoxide **9-12** was also considered. Collapse of **9-12** to 14α-oxa radical (**9-7**), followed by the aforementioned sequence **9-7→9-8→9-9**, would ultimately yield the 14β-hydroxy compound **9-6**.

Should the hypothetical 14-keto-15-radical **9-8** be sufficiently long lived to allow scrambling of the C-15 hydrogen atoms, then digitoxigenin biosynthesized from samples of [15β-^{2}H]progesterone (**10-1**) or [15α-^{2}H]progesterone (**10-2**) would be deuteriated at both the 15α *and* 15β



(10-1) $R_1 = {}^{2}H$, $R_2 = H$

(10-2) $R_1 = H$, $R_2 = {}^{2}H$

(10-3) $R_1 = R_2 = {}^{2}H$

Scheme 10.

positions. We sought to evaluate this possibility by distinguishing between 15α and 15β deuterium signals by NMR spectrometry. [15β-^{2}H]Progesterone (**10-1**), [15α-^{2}H]progesterone (**10-2**), and [15-$^{2}H_2$]progesterone (**10-3**) were synthesized and their deuterium NMR spectra

Table 1
Deuterium NMR spectral data of 15-deuterio-progesterones

[15-^{2}H]Progesterone	Chemical shift (ppm)	
(10-5)	1.66	1.22
(10-3)	1.70	
(10-4)		1.23

recorded[90] (Table 1). The deuterium signals in **10-1** and **10-2** are separated by 0.47 ppm, and the 15β and 15α deuterium signals in **10-3** are clearly resolved (though not to the baseline). We are confident that these observations will remain valid for cardenolides biosynthesized from

stereospecifically-labelled 15-deuterioprogesterones and therefore will enable isotopic differentiation of the relevant hydrogen atoms in diosgenin.

5. Stereochemistry of enzymic hydroxylation at primary carbon atoms

The steric outcome of hydroxylation at asymmetric and *pro*chiral tertiary and *pro*chiral secondary carbon atoms may be determined from the chirality of the obtained alcohols. Using substrates stereospecifically labeled with isotopic hydrogen at pertinent secondary carbon atoms, the *overall mechanism* of the enzymic process can be deduced. However, these strategies are not directly applicable to studies of stereochemical processes at primary carbon atoms.

Ogston[91–93] rationalized the course of enzymic reactions at *pro*chiral centers by proposing that a '... three-point combination occurs between the symmetrical substrate and the enzyme...'. In a *pro*chiral molecule $R_1R_2CX_aX_b$ ($R_1 \neq R_2$, $X_a = X_b$) the enzyme distinguishes between R_1 and R_2 by virtue of their size, stereochemistry, and electrostatic properties. Substituents R_1 and R_2 of $R_1R_2CX_aX_b$ fit into their respective *binding* sites, which imposes on the substrate a particular orientation within the enzyme cavity. Under these circumstances only one of the two 'identical' X moieties (either X_a or X_b) can reach the *catalytic* site of the enzyme and undergo reaction.[94]

Evaluating the steric pathway of enzymic hydroxylations at primary carbon atoms poses a more complex problem. In a freely-rotating methyl group each of the three equivalent hydrogen atoms has an equal probability of entering the *catalytic* site of the hydroxylase and being displaced. Since the resulting alcohol is also *achiral*, it is impossible to determine the steric mode of introduction of the hydroxyl moiety simply by looking at the structure of the product. The availability of procedures, however, for the synthesis of chiral methyl groups[99–102] of known configuration does render the problem amenable to solution.

The small differences in size between protium (H), deuterium (D), and tritium (T) (H > D > T) will most likely not be recognizable by an enzyme, in which case Ogston's classical formulation of stereochemical differentiation by three-point attachment between substrate and enzyme is not applicable to the analysis of enzymic transformations at chiral methyl moieties. However, should hydroxylation proceed with a sufficiently large isotope effect, the overall stereochemistry of the reaction can, in principle, be established.[103,104]

If we assume that C-1 hydroxylation of a chiral terminus of an *n*-alkane R-CTDH proceeds with an adequate *normal* primary kinetic intramolecular hydrogen isotope effect $k_H > k_D > k_T$, the hydroxylation product will be a mixture of alcohols in the relative amounts R-CTDOH > R-CTHOH > R-CDHOH. Then, by determining the chirality of the predominant tritiated alcohol derived from a chiral methyl substrate of known configuration, the overall steric course of the hydroxylation reaction may be defined.

We selected *n*-octane, which is efficiently metabolized by *Pseudomonas oleovorans* to 1-octanol, as the model substrate for our initial studies.

5.1. *Hydroxylation at chiral methyl termini of* n-*octanes*

Hydroxylation at a primary carbon atom can proceed with retention, inversion, or racemization. Options for the outcome of hydroxylation of (1*R*)[1-H,D,T]*n*-octane and (1*S*)[1-H,D,T]*n*-octane with retention or inversion and with a normal hydrogen isotope effect are summarized

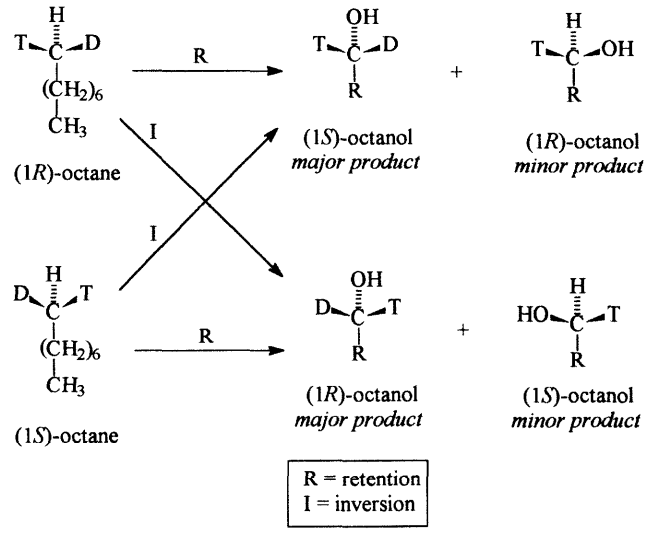


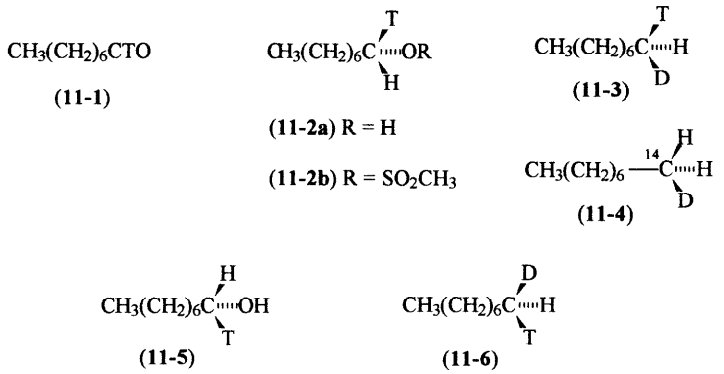
Fig. 1.

in Fig. 1. Should the reaction proceed stereospecifically, a *major* and *minor* tritiated 1-octanol will be formed in each case. The anticipated *major* tritiated products of hydroxylation of (1*R*)[1-H,D,T]*n*-octane with retention and inversion of configuration are (1*S*)-1-octanol and (1*R*)-1-octanol, respectively. In contrast, the expected *major* tritiated alcohols derived from hydroxylation of (1*S*)[1-H,D,T]*n*-octane with retention and inversion of configuration are (1*R*)-1-octanol and (1*S*)-1-octanol, respectively. Were hydroxylation to proceed with *racemization or stereospecifically in the absence of an isotope effect*, then equal amounts of tritiated (1*R*)- and (1*S*)-1-octanol would be formed *irrespective of the chirality of the substrate*.

When this work was undertaken, substrates with only relatively low specific activities of tritium could be produced. We opted to use enzymic methods of analysis, since none of the physicochemical techniques then available were sensitive enough to assay the chirality of the minute amounts of tritiated biosynthesized octanols expected. (Alcohols lacking a C-1 tritrium atom are radioisotopically invisible and therefore disregarded.)

The required substrates, (1*R*)[1-H,D,T]*n*-octane (**11-3**) mixed with [^{14}C]*n*-octane and (1*S*)[1-H,D,T]*n*-octane (**11-6**) mixed with [^{14}C]*n*-octane, were synthesized.[103,104] Reduction of [1-T]octanal (**11-1**) with NADH/horse liver alcohol dehydrogenase (HLAD), using dithionite to recycle $NAD^+ \rightarrow NADH$, gave (1*S*)[1-T]1-octanol (**11-2a**), which was then admixed with [1-^{14}C]octanol. The mixture was mesylated (**11-2b**), and the resulting mesyl esters hydrogenolyzed with $LiEt_3BD$ to give the required (1*R*)[1-H,D,T]*n*-octane (**11-3**) containing [1-D;1-^{14}C]*n*-octane (**11-4**) (i.e. (1*R*)[1-H,D,T;1-^{14}C]*n*-octane).

A portion of **11-2a** was treated with diethyl azodicarboxylate/benzoic acid/triphenylphosphine in THF,[105] and the resulting benzoate saponified. The (1*R*)[1-T]1-octanol (**11-5**) obtained was admixed with [1-^{14}C]octanol and processed as above to yield (1*S*)[1-H,D,T]*n*-octane (**11-6**) containing [1-D;1-^{14}C]*n*-octane (**11-4**) (i.e. (1*S*)[1-H,D,T;1-^{14}C]*n*-octane). While the enantiomeric purities of the synthesized chiral *n*-octanes were not determined, the results of the



Scheme 11.

enzymic hydroxylation reactions (vide infra) imply that their enantiomeric purities must have been very high.

Assuming that enzymic hydroxylation of (1*R*)[1-H,D,T;1-^{14}C]*n*-octane at the chiral terminus proceeds with retention of configuration and a normal hydrogen isotope effect, the radioactive

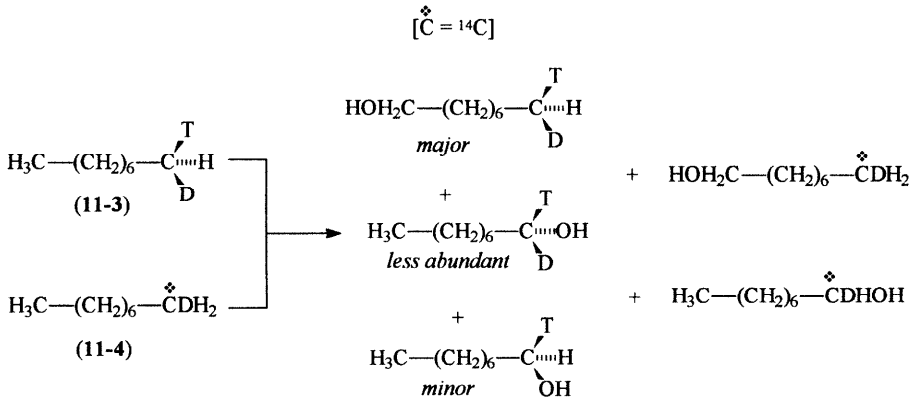


Fig. 2.

products shown in Fig. 2 will be formed in the indicated relative amounts. Hydroxylation will occur at both termini of chiral *n*-octane, but only a transformation at the chiral terminus of the molecule is germane to establishing the stereochemistry of enzymic hydroxylation at primary carbon atoms. To quantify the *extent* of enzymic attack at the chiral terminus vis-à-vis the achiral terminus, the mixture of recovered [1-T]- and [^{14}C]1-octanols would be oxidized with Jones' reagent[106] to yield a mixture of octanoic acids (Fig. 3). From the loss of tritium evidenced by changes in the T/^{14}C ratio, the percentage of hydroxylation at the chiral terminus of the octanes was calculated. The amount of tritium lost during Jones' oxidation also indicates

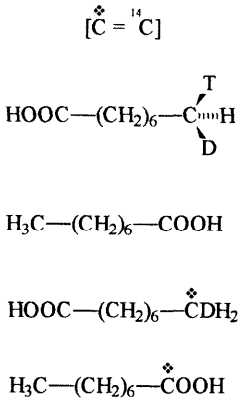


Fig. 3.

the maximal amount of tritium potentially available for abstraction in the enzymic chirality analysis of the biosynthesized 1-octanols.

We intended to determine the chirality of the biosynthesized tritiated 1-octanols by incubating them with HLAD/NAD^+, which reportedly stereospecifically abstracts the 1-*pro-R* hydrogen atom of primary alcohols to yield aldehydes. During exploratory studies, however, we discovered that (1*S*)[1-T;1-^{14}C]1-octanol was also oxidized by HLAD/NAD^+ with loss of tritium; moreover, the resultant aldehydes, [^{14}C]$C_7H_{15}CHO + C_7H_{15}CTO$, had considerably higher T/^{14}C ratios than the substrate octanols.[107] These results were rationalized by assuming that some of the produced aldehydes were further oxidized by HLAD/NAD^+ to octanoic acids[108] with a normal hydrogen isotope effect. Most likely the protiated aldehyde [^{14}C]$C_7H_{15}CHO$ was oxidized to octanoate more rapidly than the tritiated species $C_7H_{15}CTO$. Accelerated depletion of [^{14}C]$C_7H_{15}CHO$ with consequent accumulation of $C_7H_{15}CTO$ would lead to the observed increase in T/^{14}C ratio. Limited attempts to correct the situation by modifying reaction conditions were not successful, so this analytical approach was abandoned.

We next turned out attention to the HLAD/NAD(H)/porcine heart diaphorase procedure,[109,110] assumed to exchange stereospecifically the 1-*pro-R* hydrogen atoms of primary alcohols. Here, too, we observed that 1-*pro-S* hydrogen (tritium) atoms were exchanged, albeit at a much slower rate than 1-*pro-R* hydrogens. In this case we succeeded in standardizing the experimental conditions so as to yield reproducible results. It was determined that under specified conditions the 1-*pro-R* tritium atom of (1*R*)[1-T]1-octanol was almost completely exchanged with water by 12 h and *totally* exchanged by 24 h. Using (1*S*)[1-T]1-octanol and (1*RS*)[1-T]1-octanol and allowing the exchange reaction to proceed for 24 h, we observed that 30–40% of the 1-*pro-S* tritium was *consistently and reproducibly exchanged* under our experimental conditions.[107]

With a solution to the problem of the configurational analysis of C-1 chiral 1-octanols in hand, we focused on the biosynthesis of 1-octanols from chiral *n*-octanes. Samples of (1*R*)[1-H,D,T;1-^{14}C]*n*-octane and (1*S*)[1-H,D,T;1-^{14}C]*n*-octane were incubated with homogenates of the bacterium *Ps. oleovorans* strain TF4-1L, and the recovered alcohols purified. The extent of hydroxylation at the chiral termini was calculated from the loss of tritium following

Jones' oxidation of aliquots of the alcohols to octanoic acids. Other aliquots of biosynthesized 1-octanols and samples of synthetic (1*RS*)[1-T;1-^{14}C]1-octanol were separately incubated in parallel with HLAD/NAD(H)/diaphorase for 24 h. The equilibrated 1-octanols were recovered, purified, converted to 3,5-dinitrobenzoate esters, and their T/^{14}C ratios determined. The results, corrected for percentage loss of 1-*pro-S* tritium deduced from reference exchange reactions with (1*RS*)[1-T;1-^{14}C]1-octanol,[103,104] are summarized in Table 2. They show that hydroxylation at the chiral terminus proceeded to the extent of 20–30%, whereas enzymic attack occurred predominantly (70–80%) at the achiral terminus. This is consistent with the operation of a normal intramolecular hydrogen isotope effect during hydroxylation. It is also evident that hydroxylation of (1*R*)[1-H,D,T]*n*-octane and (1*S*)[1-H,D,T]*n*-octane by *Ps. oleovorans* strain TF4-1L proceeded with retention of configuration, in which the incoming hydroxyl assumed the orientation of the displaced hydrogen atom.

Table 2
Hydroxylation of (1*R*)- and (1*S*)-*n*-octanes by homogenates of *Ps. oleovorans* strain TF4-1L

Experiment	Chirality of octane	% of T at C-1 of octanols	% of (1*R*)-octanol	C-1 Chirality of the major octanol	Stereochemistry of hydroxylation
1	1*R*	26	38±2	1*S*	retention
	1*S*	22	82±6	1*R*	retention
2	1*R*	29	37±3	1*S*	retention
	1*S*	29	74±5	1*R*	retention

After establishing the stereochemistry of hydroxylation at a primary carbon atom for a bacterial system, we turned our attention to the problem of methyl hydroxylation by rat liver microsomes. Hydroxylation is a mandatory step in the metabolic disposition of many xenobiotic substances in mammals,[111–113] and occurs mainly in the liver.[114–117] A major route of elimination of *n*-alkanes in mammals is initiated by terminal hydroxylation in the liver to yield *n*-alcohols,[118–123] which are subsequently metabolized to water-soluble products. The hydroxylases of *Ps. oleovorans* and rat liver microsomes are structurally dissimilar: both are siderproteins, but whereas the liver enzyme is a *b*-type cytochrome containing protoporphyrin IX at the active site,[124] the prosthetic group of the bacterial enzyme contains nonheme iron.[125] It was therefore of great interest to compare the steric course of hydroxylation by these two analogous but non-homologous enzymes.

Samples of (1*R*)[1-H,D,T;1-^{14}C]*n*-octane and (1*S*)[1-H,D,T;1-^{14}C]*n*-octane were incubated with rat liver microsomes, and the biosynthesized 1-octanols recovered, purified, and their chiral compositions determined as described above. The results, summarized in Table 3, indicate that enzymic attack occurred mainly at the achiral terminus, demonstrating that in this system, too, hydroxylation proceeded with a normal hydrogen isotope effect. It is further clear that hydroxylation of *n*-octane by the rat liver monooxygenase proceeded with retention of configuration.[126] Thus, methyl hydroxylation in both the bacterial and rat liver microsomal systems occurred in the retention mode.

Table 3
Hydroxylation of (1*R*)- and (1*S*)-*n*-octanes by rat liver microsomes[a]

Experiment	Chirality of octane	% of T at C-1 of controls	% of (1*R*)-octanol	C-1 Chirality of the major octanol	Stereochemistry of hydroxylation
1	1*R*	25	24±2	1*S*	retention
	1*S*	29	85±6	1*R*	retention
2	1*R*	32	26±2	1*S*	retention
	1*S*	29	63±4	1*R*	retention

[a]Counted as octyl *p*-tolylurethanes.

It is worthy to note that a doctoral dissertation by A. E. Gautier[127] described results analogous to ours for hydroxylation of primary carbon atoms. Samples of (12*R*)- and (12*S*)[12-H,D,T;1-^{14}C]lauric acid were incubated with cell-free extracts of the yeast *Candida tropicalis* to yield specimens of 12-hydroxylauric acid; introduction of an hydroxyl group at C-12 of both samples proceeded in the retention mode. More recently, Floss and coworkers[128,129] demonstrated that hydroxylation of (1*R*)- and (1*S*)[1-H,D,T]ethane by the methane monooxygenases (MMOs) of some methylotrophic bacteria proceeded with a normal intramolecular kinetic hydrogen isotope effect and with net retention of configuration. These results are discussed in more detail in Section 6.

5.2. *Hydroxylation at C-19 chiral methyls of androgens*

Having established the steric course of enzymic hydroxylation at the methyl terminus of *n*-octane by both a bacterial and a mammalian microsomal system, we turned our attention to a challenging problem of considerable biochemical interest, namely the biosynthesis of estrogens from androgens. Transformation of androstenedione and testosterone to estrogens, catalyzed by a human placental 'aromatase' enzyme complex, is initiated by hydroxylation at the C-19 methyl group.[130,131] The overall process of estrogen biosynthesis from androgen proceeds in three oxidative stages, each requiring one mole of O_2 and one mole of NADPH per mole of androgen transformed[132] (Fig. 4). It has been proposed that the primary oxidation product, 19-hydroxyandrogen, undergoes a second hydroxylation at C-19 in which the 19-*pro-R* hydrogen is eliminated.[133–136] The resulting diol is thought to dehydrate to a 19-aldehyde with loss of the oxygen atom introduced during the second hydroxylation step.[136–138] A third oxygenation then takes place, and the C-19 moiety is extruded as formic acid with concomitant formation of estrogen.[139]

Two of the three C-19 hydrogens of the androgen substrate ultimately find their way into the water of the reaction medium, whilst the third hydrogen, corresponding to the 19-*pro-S* hydrogen atom of the 19-CH_2OH primary hydroxylation product, is retained in the formic acid, which also contains the two oxygen atoms introduced in the first and third oxidative steps[137,138,143] (Fig. 4).

We undertook to define the steric course of the initial C-19 hydroxylation of androgens by human placental microsomal aromatase.[144,145] As in our studies with *n*-octane, we assumed that

the stereochemistry of hydroxylation at C-19 of androgens could be determined using androgen substrates bearing a C-10 chiral methyl group (≡19-CHDT). Determining the chirality of biosynthesized tritiated 19-hydroxyandrogens is not feasible with the alcohol dehydrogenase/NAD(H)/diaphorase exchange reaction which proved so useful for the analysis of biosynthesized 1-octanols. Therefore we adopted an indirect approach predicated on established aspects of estrogen biosynthesis.

Hydroxylation of steroidal C-19 methyl groups can proceed either with retention, inversion, or racemization. Hydroxylation of, e.g., (19*R*)[19-H,D,T]3β-hydroxyandrost-5-en-17-one with a normal hydrogen isotope effect in either the retention or inversion mode will yield the products shown in Fig. 5 in the indicated qualitative proportions; reciprocal results are to be expected for

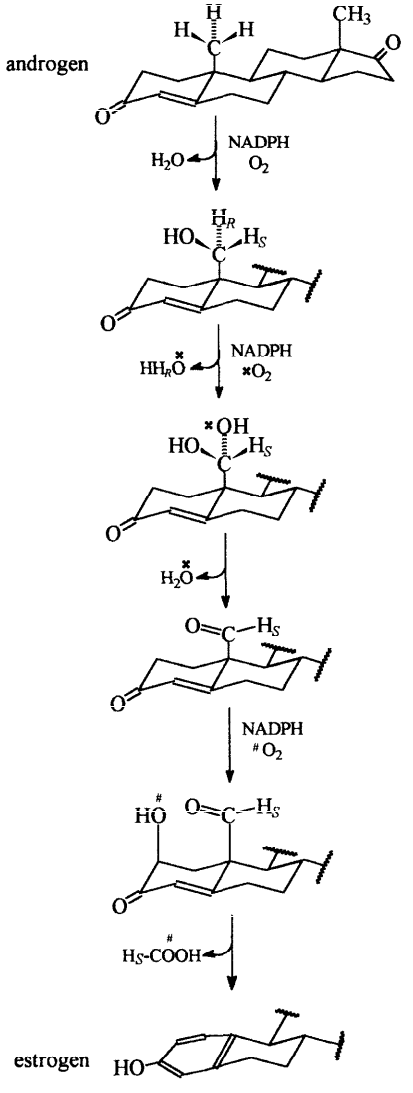


Fig. 4.

the (19*S*)-analogue. The products of hydroxylation of a (19*S*)-substrate in the inversion mode correspond to those obtained by hydroxylation of a (19*R*)-substrate in the retention mode, whereas products obtained by hydroxylation of a (19*S*)-substrate with retention of configuration are the same as those derived by hydroxylation of a (19*R*)-compound with inversion of configuration.

As previously mentioned, initial hydroxylation at the C-19 methyl moiety is followed by a second hydroxylation at C-19; introduction of this second 19-hydroxyl group proceeds *without* an isotope effect.[146] Dehydration of the 19-*gem*-diol results in a 19-oxoandrogen, which is then converted via reaction with the third mole of O_2+NADPH to estrogen and formic acid. The sequence of events subsequent to primary hydroxylation of (19*R*)-androgen with retention of configuration is presented in Fig. 6. At the end of this process much more tritium should be found in formic acid than in water. Conversely, were initial hydroxylation of (19*R*)-androgen to take place with inversion of configuration, more tritium should be found in water than in formic acid.

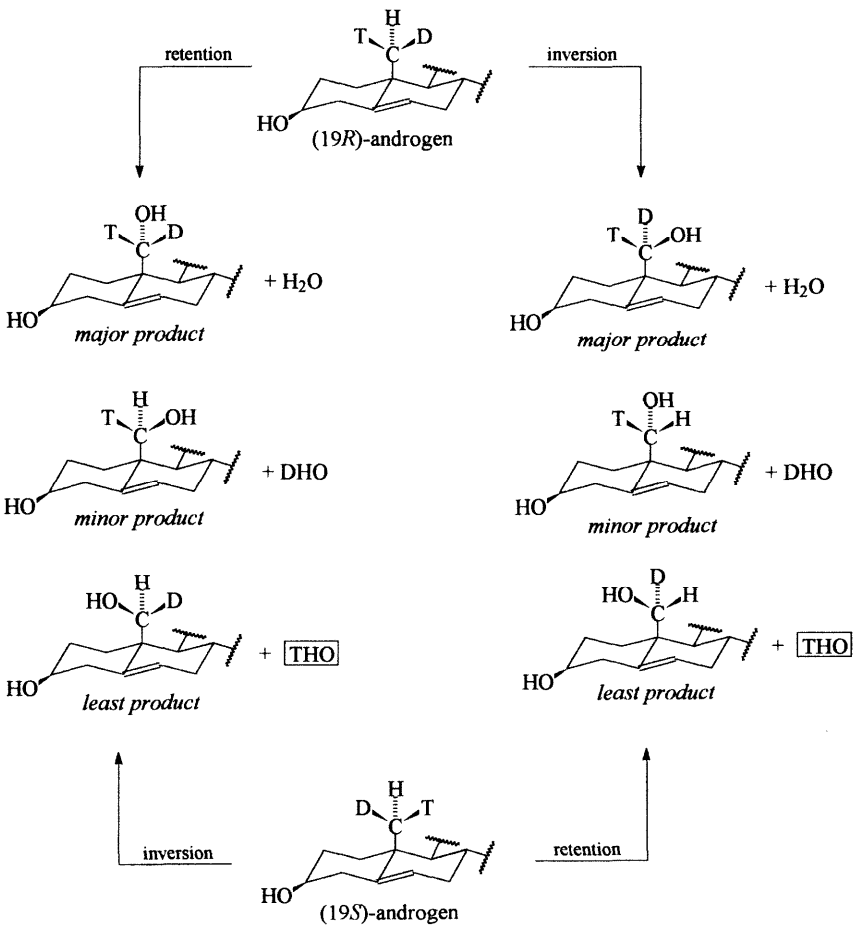


Fig. 5.

The reasoning is equally applicable to (19*S*)-androgen, for which hydroxylation in the retention mode should result in the accumulation of more tritium in water than in formic acid; whereas hydroxylation of a (19*S*)-substrate in the inversion mode should lead to formic acid containing more tritium than what would be found in water.

Were initial hydroxylation to occur with an isotope effect but with racemization, the expected results are as depicted in Fig. 7. If the first hydroxylation of (19*R*)-androgen proceeds with 50% retention and with 50% inversion, the isotope effect for both processes will have been the same. If the second hydroxylation proceeds without an isotope effect[146] and the 19-hydroxy intermediates are completely converted to estrogen, then at the completion of aromatization the total amount of tritium retained by the formic acid must equal the total amount found in water:

$$[\mathrm{TCOOH}] = [\mathrm{THO}] \tag{1a}$$

or

$$[\mathrm{TCOOH}]/[\mathrm{THO}] = 1 \tag{1b}$$

In the unlikely event that the first hydroxylation were to proceed with a negligible isotope effect, a significant amount of tritium will be released in the form of water. Under these

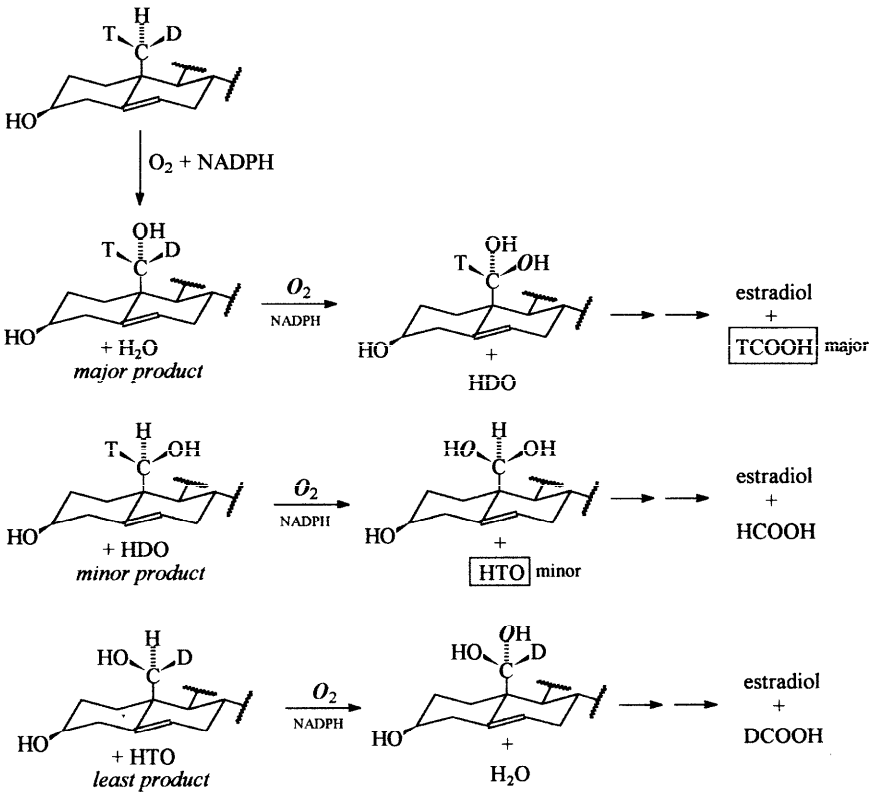


Fig. 6.

circumstances considerably more tritium will occur in water than in formic acid, and the ratio of tritium in formic acid to tritium in water will be significantly less than one:

$$[\mathrm{TCOOH}]/[\mathrm{THO}] \ll 1 \tag{2}$$

However, if the initial hydroxylation involves a normal isotope effect, only small amounts of tritiated water will be produced and the ratio will be significantly greater than one:

$$[\mathrm{TCOOH}]/[\mathrm{THO}] \gg 1. \tag{3}$$

If the first hydroxylation step does *not* involve an isotope effect, then irrespective of the C-19 chirality of the substrate (19*R*, 19*S*, 19*RS*) and mode of hydroxylation (retention, inversion, racemization) all three isotopes of hydrogen will be abstracted with equal frequency. The sequence of events for hydroxylation with retention of configuration but without an isotope effect is illustrated for (19*R*)-androgen in Fig. 8, in which it will be seen that aromatization is accompanied by the appearance of twice as much tritium in water than in formic acid:

$$[\mathrm{THO}]/[\mathrm{TCOOH}] = 2. \tag{4}$$

Identical results are to be expected for the (19*S*)- and (19*RS*)-androgens.

Thus, by knowing the C-19 chirality of the androgen incubated with placental aromatase, the steric course of the first C-19 hydroxylation can be ascertained from the relative amounts of tritium in the recovered formic acid and water.

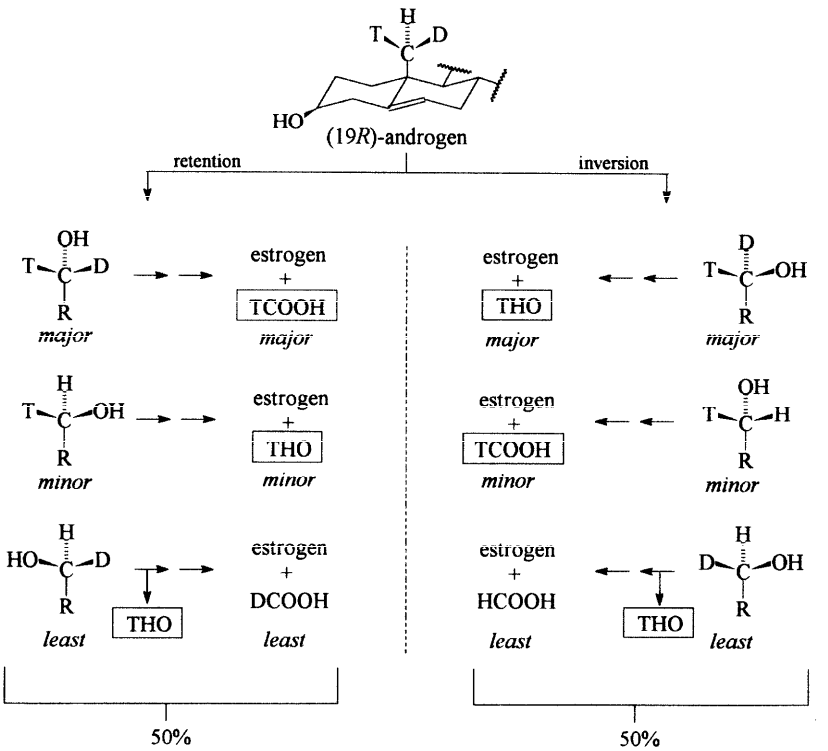


Fig. 7.

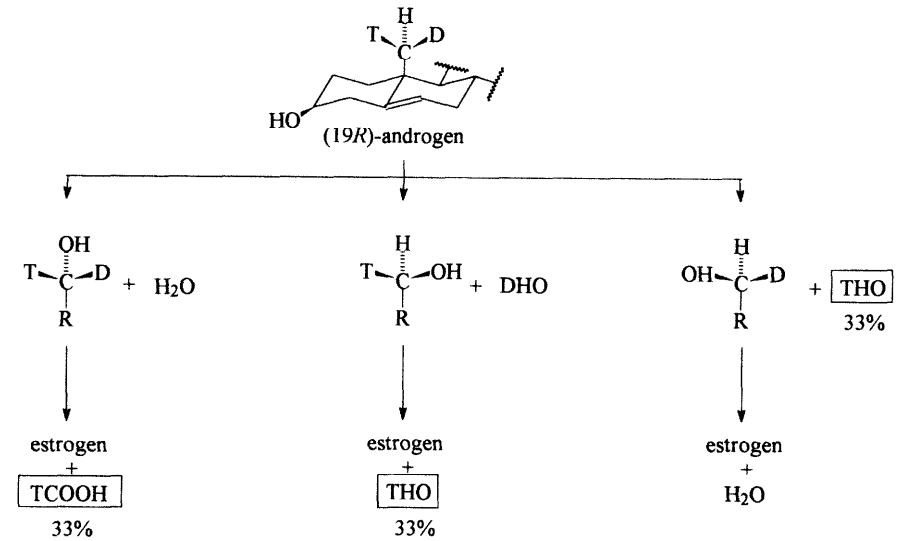


Fig. 8.

Having conceptualized a solution for determining the stereochemistry of the first C-19 hydroxylation during androgen conversion to estrogen, we focused our attention on the synthesis of the requisite C-19 chiral substrates.[147,148]

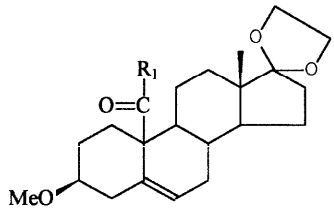
Two syntheses of C-19 chiral androgens were developed, based on the stereochemically-controlled introduction of the three isotopic hydrogen atoms in the construction of the 10β methyl group. In exploratory studies we found that reduction of [19-D]17,17-ethylenedioxy-3β-methoxyandrost-5-en-19-al (**12-1a**) with Haubenstock reagent[149] [$Li(Bu_2CHO)_3AlH$] gave a mixture of alcohols consisting of 90–95% (19*R*)-alcohol (**12-2**) and 5–10% (19*S*)-alcohol (**12-3**), whereas when protiated aldehyde (**12-1b**) was reduced with deuteriated Haubenstock reagent the recovered mixture contained 5–10% (19*R*)-alcohol (**12-2**) and 90–95% (19*S*)-alcohol (**12-3**).

From these observations the following sequence of reactions was developed. To simplify discussion of the syntheses we disregard the minor components produced. The 19-tritiated aldehyde **12-1c** was reduced with protiated Haubenstock reagent. Following removal of the 17-ketal group the resulting (19*R*)-hydroxy-17-keto compound (**12-4**) was converted with retention of configuration to (19*R*)-iodide (**12-5**) by treatment with methyltriphenoxyphosphonium iodide in dry DMF.[150,151] Hydrogenolysis of the iodide with $LiEt_3BD$ and reoxidation gave the (19*S*)-compound **12-6a**, cleavage of whose C-3β methoxyl moiety provided the required (19*S*)-3β-hydroxyandrost-5-en-17-one (**12-6b**). Synthesis of the corresponding (19*R*)-androgen was achieved essentially as previously described, except that tritiated aldehyde **12-1c** was reduced with deuteriated Haubenstock reagent to yield **12-7**, and iodide **12-8** was hydrogenolyzed with $LiEt_3BH$ to give **12-9a**. Cleavage of the 3β-methoxyl group yielded (19*R*)-androgen **12-9b**.

The second synthesis again starts with 19-tritiated aldehyde **12-1c**. This aldehyde was reduced with (*R*)-[^{2}H]-Alpine-Borane® or (*S*)-[^{2}H]-Alpine-Borane® to give (following removal of the 17-ketals) (19*S*)-alcohol **12-10** or (19*R*)-alcohol **12-7**, respectively. The derived (19*S*)-iodide

12-7, respectively. The derived (19*S*)-iodide **12-11** and (19*R*)-iodide **12-8** were each hydrogenolyzed with $LiEt_3BH$ and processed to give the (19*S*)-derivative **12-6a** and (19*R*)-derivative **12-9a**. Hydrolysis of the 3β-methoxyl moieties provided the required (19*S*)-androgen **12-6b** and (19*R*)-androgen **12-9b**.

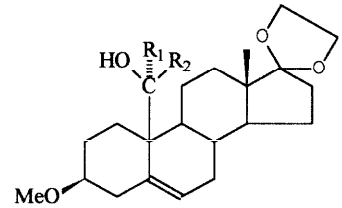
At this point the configurations of the C-19 chiral methyl moieties of the synthesized androgens were not known. The chiralities of the C-19 alcohols and C-19 iodides had been firmly established by NMR spectrometry, but no information was available on the stereochemical course of hydrogenolysis of iodides with $LiEt_3BH$. To determine the C-19 chirality of the four samples of synthesized androgens we intended to subject them to Kuhn–Roth oxidation, recover the acetic acids thus formed, and assay their chiralities with malate synthase/fumarase.[100–102] However, under conventional conditions of Kuhn–Roth oxidation some 20–30% of acetate methyl hydrogens were exchanged.[152] An alternative two-step oxidation procedure was therefore developed, in which a *maximum* of 5% of acetate methyl hydrogen atoms were exchanged.[152] The synthesized 19-chiral androgens were oxidized by this alternative method and the isolated acetic acids assayed with malate synthase/fumarase. The results are summarized in Table 4. The *F*-values of the acetates, and hence the C-19 chiralities of the androgens from which the acetates were obtained, were the same for the two (19*S*)-androgens



(12-1a) R_1 = D

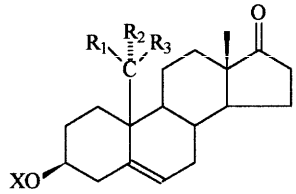
(12-1b) R_1 = H

(12-1c) R_1 = T



(12-2) R_1 = D, R_2 = H

(12-3) R_1 = H, R_2 = D



(12-4) R_1= OH, R_2 = T, R_3 = H, X = Me

(12-5) R_1 = I, R_2 = T, R_3 = H, X = Me

(12-6a) R_1 = D, R_2 = H, R_3 = T, X = Me

(12-6b) R_1 = D, R_2 = X = H, R_3 = T

(12-7) R_1 = OH, R_2 = T, R_3 = D, X = Me

(12-8) R_1 = I, R_2 = T, R_3 = D, X = Me

(12-9a) R_1 = T, R_2 = H, R_3 = D, X = Me

(12-9b) R_1 = T, R_2 = X = H, R_3 = D

(12-10) R_1 = OH, R_2 = D, R_3 = T

(12-11) R_1 = I, R_2 = D, R_3 = T

Scheme 12.

Table 4
F-Values and calculated chiralities of 19-chiral 3β-hydroxyandrost-5-en-17-ones

Androgen	*F*-value	Calculated chiral composition	Corrected chiral composition
19*R*	64	76% 19*R*+24% 19*S*	81% 19*R*+19% 19*S*
19*S*	33	80% 19*S*+20% 19*R*	85% 19*S*+15% 19*R*

12-6b and for the two (19*R*)-androgens **12-9b** produced by the different synthetic procedures. Assuming a linear relationship between the *F*-value and enantiomeric purity, it was inferred that the (19*R*)-androgens **12-9b** each contained 76% of (19*R*)-methyl moieties and that the (19*S*)-androgens **12-6b** each contained 80% of (19*S*)-methyl moieties. Since the oxidation procedure results in exchange of up to 5% of the hydrogen atoms of the acetate methyl groups, it may be further calculated that the (19*R*)-androgens **12-9b** each contained up to 81% of (19*R*)-methyl moieties, whereas the (19*S*)-androgens **12-6b** each contained up to 85% of (19*S*)-methyl moieties. These results also indicate that hydrogenolysis of the iodide derivatives with $LiEt_3BH$ proceeded with inversion of configuration.

Equipped now with the necessary substrates and an analytical methodology, we undertook to determine the steric mode of the first C-19 hydroxylation of androgens by placental aromatase. Samples of (19*R*)-, (19*S*)-, and (19*RS*)[19-H,D,T]3β-hydroxyandrost-5-ene-17-one (100 μg, 2.1 μCi of T) were admixed with [4-^{14}C]3β-hydroxyandrost-5-en-17-one (35 μg, 1 μCi of ^{14}C) and aerobically incubated with an aromatase preparation (1 h, 35–37°C). Experiments were performed in triplicate using aliquots from the same batch of aromatase. Reactions were terminated by acidification with phosphoric acid, then reaction mixtures frozen in liquid nitrogen and lyophilized. Freeze-dried residues were reconstituted with water and the steroids recovered with ethyl acetate. The ethyl acetate extracts were partitioned with 1.25 M NaOH, and the alkaline solution processed in the conventional manner to yield crude phenolic fractions. The phenolic fractions were treated with $NaBH_4$, resolved by HPLC, and the samples of estradiol isolated.

The 'distillate' from each lyophilized reaction mixture was alkalized, frozen, and lyophilized again. The final 'volatile fraction' contained THO. The TCOONa remained in the residue, and

Table 5
Hydroxylation of (19*R*)-, (19*RS*)-, and (19*S*)-3β-hydroxyandrost-5-en-17-ones by human placental microsomal aromatase

Experiment	Chirality of androgen	[TCOOH/THO]	Stereochemistry of hydroxylation
1	19*R*	1.45	retention
	19*RS*	0.94	
	19*S*	0.68	retention
2	19*R*	1.47	retention
	19*RS*	0.97	
	19*S*	0.70	retention
3	19*R*	1.41	retention
	19*RS*	0.92	
	19*S*	0.69	retention

was counted as benzyl formate. The results, shown in Table 5, are corrected for recovery

of the relevant components as per control experiments. The percentage conversion of [19-H,D,T]androgen into estrogen was calculated from the sum of tritium in TCOOH and THO. The percentage of [4-^{14}C]androgen aromatized was determined from the quantity of [^{14}C]estradiol recovered. In all cases the amount of estradiol derived from [4-^{14}C]androgen exceeded by about 20% that derived from the [19-H,D,T]-substrate. The turnover ratio of [4-^{14}C;19-H_3]androgen versus [19-H,D,T]androgen is 1.24 and should approximate the *overall* hydrogen isotope effect $k_H/(k_H,k_D,k_T)$. Although this value is somewhat low, it nonetheless indicates that initial hydroxylation at C-19 indeed proceeds with a normal intramolecular hydrogen isotope effect.

That the average [TCOOH]/[THO] ratio for the (19*RS*)-substrate was 0.94 (Table 5) shows that there was almost an equal distribution of tritium between water and formic acid, as expected for racemic C-19 androgen. The formic acid recovered from placental incubations with (19*R*)-androgen contained more tritium than the water (average [TCOOH]/[THO] = 1.44). In contrast the water recovered from placental incubations with (19*S*)-androgen contained more tritium than the formic acid (average [TCOOH]/[THO] ratio of 0.69). These results are consistent with the view that initial hydroxylation at C-19 of androgens proceeds with retention of configuration.

It was previously mentioned that the enantiomeric purities of the synthesized [19-H,D,T]-androgens were nearly the same, each enantiomer constituting ca. 80–85% of molecules with the indicated chiral methyl moiety. It is therefore noteworthy that *reciprocal* results were obtained for the (19*R*)- and (19*S*)-androgens (1.44 = 1/0.69), since reciprocal results are expected if aromatization of the 19-hydroxy intermediate goes to completion or if the second 19-hydroxylation proceeds without an isotope effect.[146] Equal amounts of C-19 chiral substrate were used in all incubations; the fact that essentially equal amounts of tritium (TCOOH + THO) were released in all experiments is consistent with the view that once the initial C-19 hydroxylation takes place the molecule becomnes committed to aromatization.

6. Mechanistic implications of the steric course of hydroxylation at primary carbon atoms

Rections catalyzed by monooxygenases at unactivated sp^3 carbon atoms exhibit different regioselectivities and stereoselectivities, depending upon the source of the enzyme and the nature of the substrate. It has been suggested[153] that the prosthetic group controls the basic mechanism of the reaction while the apoprotein largely controls regio- and stereoselectivity. The issue of regioselectivity per se was not addressed in our studies, but a few words on the subject are warranted.

n-Octane,[104,126] lauric acid,[127] and geraniol[154] each offer primary and secondary carbon atoms as prospective hydroxylation sites, while the androgens[145] additionally afford tertiary carbon atoms as prospective sites for hydroxylation. The aim of our work was to define events at primary carbon atoms.[155] Barring unexpected migration of radioisotope to other sites the substrates employed were labeled with tritium *only* at primary carbon atoms.

In terms of simple chemical reactivity, the relative ability of a C–H bond to undergo hydroxylation may be estimated from the relative stability and ionization potential of the resulting radical[159] (vide infra), which for hydrogen abstraction generally follows the order

tertiary > secondary > primary. However, enzymic factors can override chemical reactivities, as illustrated by cytochrome $P_{450LA\omega}$ from clofibrate-induced rat liver, the active site of which is so structured as to suppress $(\omega-1)$-hydroxylation of lauric acid in favor of the chemically less reactive methyl terminus.[160]

Hamilton[161] postulated that monooxygenases interact with O_2 to form electrophilic 'oxenoid' species capable of inserting a singlet oxygen into a C–H bond to produce an alcohol (see ref. 162). Oxenoid transfer was envisioned as proceeding by attack of electrophilic oxygen $[O]_e$ at a σ-bond to generate a triangular 'Skell–Doering transition state'[163–165] which collapses to product with retention of configuration. Recent calculations[166] have indicated that high energy electrophiles such as OH^+ react with methane without a barrier so that direct C–H insertion or carbon attack pathways are not differentiated, though the most favorable binding arrangements involve OH^+ bound directly to the carbon atom.

A decade after Hamilton's proposal, similarities were noted between hydroxylations at aliphatic carbons by Fenton's reagent and P_{450}-catalyzed monooxygenations[167–171] (see also refs 172–175). This led to suggestions that the rate-determining step in the hydroxylation process is a homolytic hydrogen abstraction, in which $[O]_e$ is a ferryl ion-like species containing pentavalent iron ($Fe^{5+}=O$) obtained by dehydration of Fe^{3+}-OOH. According to this scheme, the position to be hydroxylated is 'selected' by virtue of its proximity and geometry vis-à-vis the ferryl oxygen atom. Abstraction of a substrate hydrogen by ferryl oxygen results in the formation of an hydroxyferryl species, Fe^{4+}-OH, and a substrate carbon radical. Thermal cleavage of Fe^{4+}-OH accompanied by electronic rearrangement yields Fe^{3+} --- • OH, combination of which with the carbon radical gives the oxygenated product and regenerates ferric P_{450}, ready to commence another round of catalysis. It was subsequently proposed[176] that dissociation of Fe^{4+}-OH prior to hydroxylation is unlikely, and that hydroxylation involves a bimolecular homolytic substitution (S_H2) at oxygen of carbon for iron (Fig. 9).

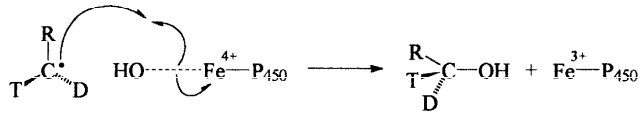


Fig. 9.

Mechanisms of oxygenation at unactivated carbon atoms were the subject of semi-empirical quantum mechanical calculations,[177] which predicted a deuterium isotope effect of ~1.6 for singlet (^1D) oxygen insertion and 8–9 for the triplet (^3P) oxygen reaction proceeding by the so-called 'oxygen rebound' mechanism. In fact, these values correspond well to the overall values found for hydroxylation at the methyl termini of *n*-octane[178,179] and geraniol.[180] Intramolecular deuterium isotope effects on the order of 4–5 have been obtained for hydroxylation of (1*R*)- and (1*S*)[1-H,D,T]ethane by methylotrophic bacteria.[128,129]

As mentioned earlier, the overall isotope effect observed for hydroxylation of C-19 chiral methyl groups of androgen, 1.24, a 'composite' isotope effect for several reactive steps in the transformation of androgen to estrogen, is relatively low. However, the intrinsic isotope effect on C–H bond breakage can be significantly or even totally masked by other rate factors in an enzymic process.[181] Some studies suggest that hydroxylation at the C-19 methyl moiety of

androgen proceeds with a relatively small tritium isotope effect ($k_H/k_T \sim 3$)[182] (see also refs 183–185). Our results on the distribution of tritium extruded from the C-19 methyl moiety of [19-H,D,T]androgens into formic acid and water are only consistent with the operation of a relatively large hydrogen isotope effect during the 'first' hydroxylation reaction. This lends further credence to speculation[153] that all P_{450} reactions proceed by stepwise radical mechanisms. Homolytic cleavage of a C–H bond in a chiral methyl moiety will give rise to a chiral radical pair assembly or a chiral radical-hydroxyferryl assembly. In either case, homolysis of the C–H bond at the chiral terminus of, e.g., (1*R*)[1-H,D,T]*n*-octane generates an assembly in which the *si* face of the planar 1-octanyl radical is oriented towards the hydroxyl radical;

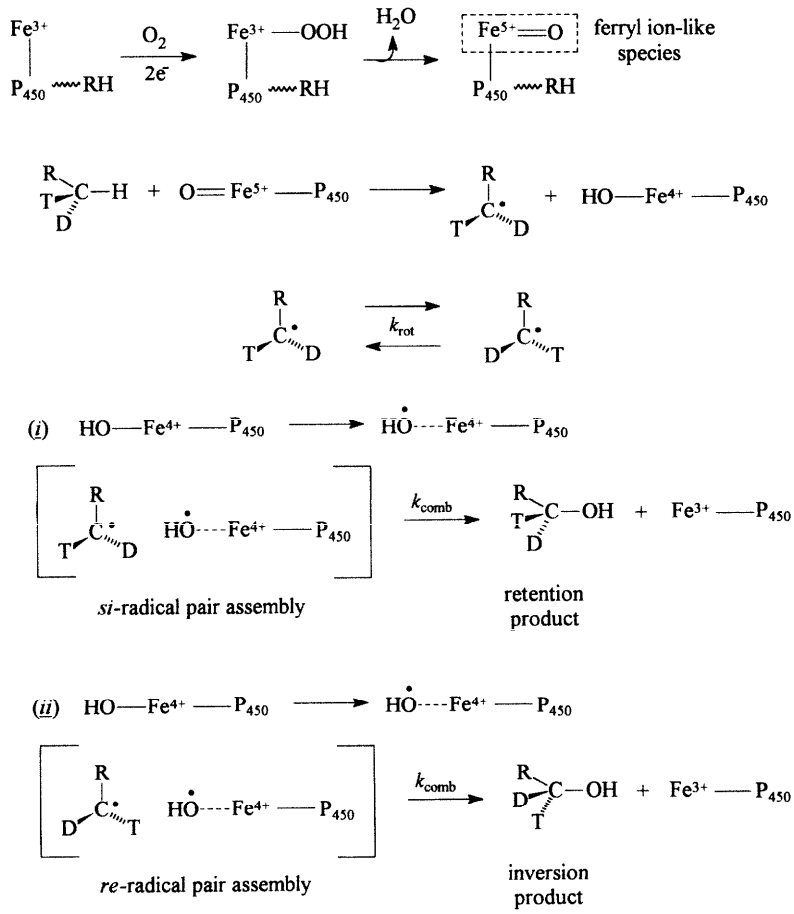


Fig. 10.

combination of this assembly yields the retention product (1*S*)[1-D,T]1-octanol (Fig. 10). In the absence of mitigating circumstances, a primary carbon radical can be reasonably assumed to be torsiosymmetric, and an out-of-plane 180° rotation about the R–C· axis will produce the enantiomeric assembly with the *re* face of the 1-octanyl radical directed towards the hydroxyl species. Combination of this assembly will yield the inversion product, (1*R*)[1-D,T]1-octanol.

The stereochemical outcome of the reaction will depend upon the relative rates of radical rotation k_{rot} and assembly combination k_{comb} within the active site of the hydroxylase. Since delivery of oxygen to primary carbon atoms occurs with a discernible stereospecificity, namely retention of configuration, it stands to reason that $k_{rot} \leq k_{comb}$. These results are consistent with the observation that 'cage' reactions initiated by single-bond homolyses proceed with a high degree of retention of configuration.[186,187] The best value to date of k_{comb} for P_{450}-catalyzed reactions is estimated[188] to be on the order of $1.5 \times 10^{13}\ s^{-1}$. A value for k_{rot} can be calculated from the Arrhenius rate equation $k = Ae^{-(Ea/RT)}$. If *n*-alkyl radicals have C–C· rotational barriers (E_a) of ~0.15 kcal/mol,[189] and if the corresponding value[190] of the Arrhenius pre-exponential factor (A) is $10^{12.5}$, then at 37°C the value of $k_{rot} \sim 3 \times 10^{12}\ s^{-1}$. Since k_{comb} is estimated to be some five times greater than k_{rot}, were substitution of hydrogen by hydroxyl to proceed with an infinite intramolecular hydrogen isotope effect and with 100% stereoselectivity, 15–20% of inversion product would be expected simply from substrate rotation about the R–C·axis. Because 20–35% of inversion product is obtained from incubation of chiral *n*-octanes with cell-free extracts of *Ps. oleovorans*, and 15–35% from incubation with rat liver microsomes, hydroxylation must proceed within the confines of the enzyme (Fig. 10) both with a large intramolecular hydrogen isotope effect and with a high degree of stereoselectivity. That abstraction–combination proceeds within an enzyme and/or solvent 'cage' is also significant in so far as it explains why these ubiquitous biochemical processes are also not toxication reactions liberating carbon-centered free radicals.[192]

In 1992, Floss and coworkers reported on the hydroxylation of (1*R*)- and (1*S*)[1j-H,D,T]-ethane by the soluble MMO of *Methylosinus trichosporium* OB3b. Using substrates with very high tritium specific activities and examining recovered ethanols by tritium NMR spectrometry, they obtained results quantitatively very similar to those obtained by our group more than a decade earlier,[103,104] namely (i) hydroxylation proceeds with a normal intramolecular kinetic hydrogen isotope effect, (ii) hydroxylation proceeds with some degree of inversion (*M. trichosporium*: 65–70% retention + 30–35% inversion; *Ps. oleovorans*: 65–80% retention + 20–35% inversion), and (iii) hydroxylation proceeds with an overall retention of configuration. A quantum chemical analysis of this system[193] led to the very provocative suggestion that, at least in the course of ethane hydroxylation by the *M. trichosporium* soluble MMO, a weak covalent bond forms between the ethyl moiety and an iron atom in the active site of the enzyme. As depicted in Fig. 11, transient ethylation of the MMO active site involves two steps, neither of which alters the stereochemistry of the substrate. Of course, it may be argued that this mechanism can accommodate sequential inversions of the Fe–C reactions leading to hydroxylation with net retention of configuration. Whilst the available data do not automatically preclude such a 'double inversion' mechanism, the proposed hydrogen abstraction and radical combination with active site iron[193] can almost certainly be regarded as proceeding within a cage formed by the protein active site, in which case there is little opportunity for a 'double inversion'.

Very recent experiments by Floss and coworkers[129,194] indicate that, within the limits of experimental error, hydroxylation of chiral methyl moieties by the particulate methane monooxygenase of *Methylococcus capsulatus* (Bath) proceeds with 100% stereoselectivity. They conclude that hydroxylation by this particular system occurs much too rapidly to be explainable by discrete abstraction–combination steps, and instead propose a concerted mechanism[188] in

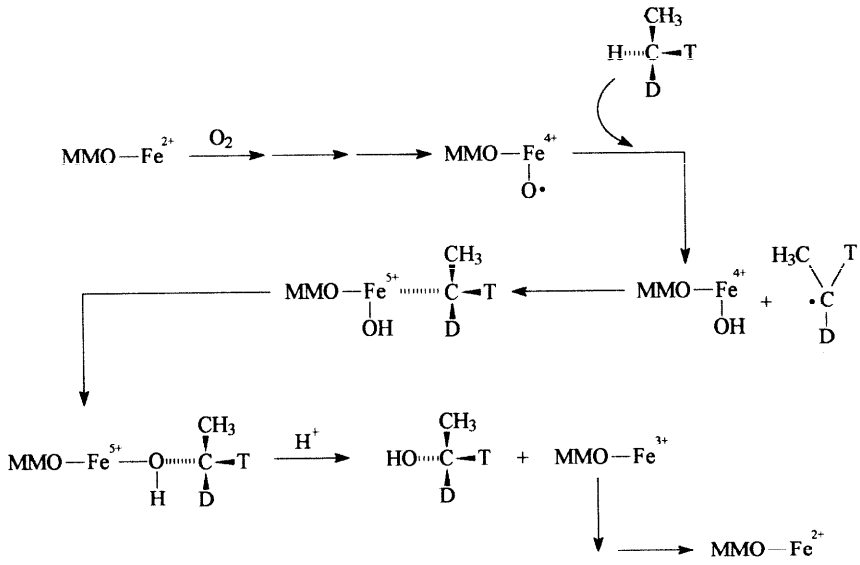


Fig. 11.

which a 'side-on' (as opposed to 'head-on') approach of the reactive oxygen atom to within bonding distance of the C–H bond leads to the formation of a pentacoordinated carbon species reminiscent of a Skell–Doering transition state, which subsequently decomposes to alcohol and monooxygenase with retention of configuration (Fig. 12).

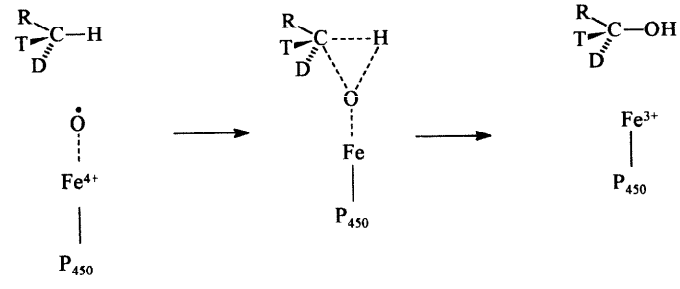


Fig. 12. P_{450}-catalysed hydroxylation via "side-on" approach of reactive oxygen, after [188].

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Reactions with the indicated stoichiometry are in fact known, but these reportedly proceed with retention of configuration. See Sawaki, Y. *Epoxidation and Hydroxylation*. In: Patai, S., Ed. The Chemistry of Hydroxyl, Ether and Peroxide Groups. Chichester, UK, John Wiley & Sons, 1993, 587–656.
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    In contrast to 17-keto precursors, aromatization of which proceeds with the stereospecific removal of the 1β- and 2β-hydrogen atoms, aromatization of 17-hydroxyandrogens and 19-norandrogens is stereospecific soley with respect to removal of the 1β-hydrogen atom.[143] In the latter substrates both the 2α- and 2β-hydrogen atoms are removed in variable amounts.[143]
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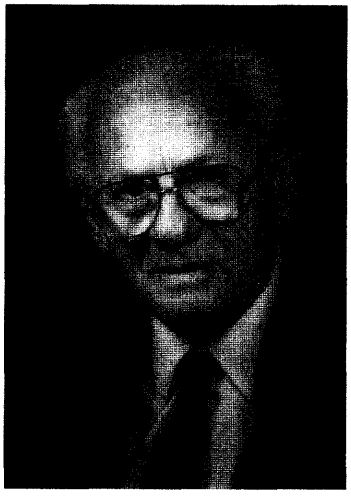
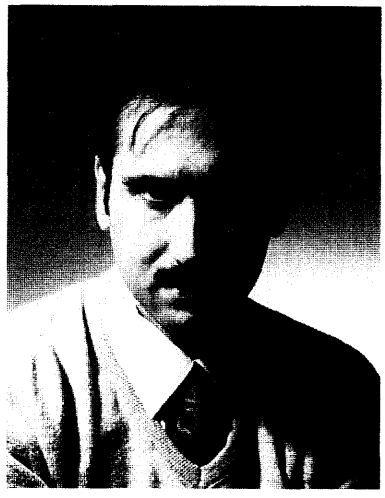
Biographical Sketch



E. Caspi



S. Shapiro

Eliahu Caspi. I was born on 10 June 1913 in Warsaw, Poland, and in 1932 graduated from Gymnazium Ascola with a Matura certificate. My subsequent studies at the University of Paris were interrupted because of illness in my family. I returned to Warsaw and enrolled in the Chemistry Department in Warsaw University, and in 1939 I completed my graduate studies with a thesis on 'Anodic oxidation of ferrochrome' under Prof. M. Centnerszwer in the Institute of Physical Chemistry. The outbreak of World War II disrupted my studies. I escaped from Warsaw, only to be trapped by the Soviets who invaded Poland from the east. I was arrested on the Polish–Rumanian border by Soviet border guards, charged with trying to escape the Soviet Union, and administratively sentenced to seven years of hard labour. I was shipped to a prison labour camp in the Arctic region of the Pechora River. Following the amnesty of Polish citizens, I was released from the prison camp and made my way to Israel. I worked as a Staff Scientist at the Standards Institute of Israel in Tel-Aviv. My military service was in the Hagana Defence Forces (1943–1947), and in the War of Israeli Independence. I was assigned to the Science Division of the Israeli Army (1947–1950). In 1950 I came to the United States to complete my interrupted education and enrolled in the graduate school of Clark University (Worcester, MA, USA) from which I received a Ph.D. degree in Chemistry (1955) for a thesis entitled 'Studies of transformation products resulting from the perfusion of cortisone and cortisol through rat livers'. Since 1951 I have been a member of the faculty of the Worcester Foundation for Biomedical Research (formerly the Worcester Foundation for Experimental Biology) in Shrewsbury, MA. There I progressed from Scientist (1951–1957) to Staff Scientist (1957–1962), Senior Scientist (1962–1971), Principal Scientist (1971–1992), and finally Principal Scientist Emeritus (1992–present day). I have been awarded the Gregory Pincus Medal; A. C. S. (MA division) Award for Distinguished Accomplishments in Organic Chemistry; N. I. H. Cancer Institute Career Development Award, and numerous visiting professorships and lectureships. My scientific interests focused on the biosynthesis of polyprenoids (sterols, protosterols, triterpenes, cardenolides, etc.); synthesis and biosynthesis, mode of action, and metabolism of steroid hormones; and stereochemistry of enzymic reactions. The work in my department has resulted in 263 publications to date.

S. Shapiro received a Ph.D. in Life Sciences (specialization in bio-organic chemistry) from Worcester Polytechnic Institute/Worcester Foundation for Experimental Biology, Inc. under the direction of Prof. E. Caspi. After completing post-doctoral studies on actinomycete physiology and antibiotic biosyntheses with Prof. L. C. Vining

(Dalhousie University, Halifax, Nova Scotia, Canada), he was recruited to head the Industrial Microbiology Laboratory at Sigma-Tau Industrie Farmaceutiche Riunite S.p.A. (Pomezia, Italy). Since 1991 Dr Shapiro has been on the staff of the Institut für orale Mikrobiologie and allgemeine Immunologie, Zentrum für Zahn-, Mund- und Kieferheilkunde der Universität Zürich, where his current research focuses on microbial physiology, natural products, quantum chemistry, and molecular modelling and quantitative structure–activity relationships of antimicrobial compounds. Dr Shapiro, who holds dual Swiss–American citizenships, resides in Kilchberg (ZH) with his wife Corine (née Bloch) and their children Noah and Ilja.