Effect of the Configuration of the Methyl Group at C-4 on the Capacity of 4-Methyl-9β, 19-Cyclosteroids to be Substrates of a Cyclopropane Cleavage Enzyme from Maize[1]

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ABSTRACT

The cyclopropane ring of 4α,14α,(24ξ)-24-trimethyl-9β19-cyclo-5α-cholestane-3β-ol is opened by microsomes isolated from maize embryos to yield 4α,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3β-ol. In contrast, when 4β,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3β-ol or 4,4,14α(24ξ)-24-tetramethyl-9β,19-cyclo-5α-cholestane-3β-ol are used as substrates, no reaction takes place. These results show that the number and also the stereochemistry of the C-4-methyls control the enzymic reaction. A new synthetic method for preparing 4β-methyl from 4α-methyl sterols is also described.

INTRODUCTION

A number of 4α-methyl-9β,19-cyclosteroids have been isolated and detected in plants (1). Notable examples include cycloeucalenol (I), 31-nor cycloartenol (2), and the buxus alcaloids (3). The only evidence of naturally occuring 4β-methyl-9β,10-cyclosteroids was the reported isolation of cyclobuxamine-H from *Buxus sempervirens* (4). Later Nakano et al. (5) and Voticky and Paulik (6) demonstrated an α configuration for the 4-methyl of cyclobuxamine-H. There is still no example of the presence of a 4β-methyl-9β,-19-cyclosteroid in nature. Cycloeucalenol (I) plays a key role in the biosynthesis of phytosterols since it appears to be the substrate of an enzyme capable of opening the 9β,19-cyclopropane ring. This has been demonstrated by incubation of I in the presence of microsomes from branble (*Rubus fruticosus*) tissue cultures (7) or from maize (*Zea mays*) embryos (8). In these conditions cycloeucalenol (I) yields obtusifoliol (II), another presumed intermediate in phytosterol biosynthesis (7). In the same conditions, 24-methylene cysloartanol (III), a 4,4-dimethyl-9β,19-cyclosteroid, is not transformed. These results suggest that the presence of two methyls at C-4 in place of a 4α-methyl inhibits the action of the enzyme (7,8), and that the 4β-methyl group could be responsible for the inhibition. Thus it could be interesting to use a 4β-methyl-9β,19-cyclosteroid as a substrate in an enzymatic preparation capable of opening the cyclopropane ring of cycloeucalenol. This communication reports the chemical synthesis of 4β,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3β-ol (IV), compares some spectroscopic properties of IV with those of the 4α-methyl epimer (V), and reports the enzymatic convertibility of IV into 4β,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3β-ol (VI).

EXPERIMENTAL PROCEDURES

Thin layer chromatography (TLC) was performed on 20x20-cm plates coated with 0.2 mm of silica gel (MERCK). Analytical plates were sprayed with the vanillin/H_3PO_4 reagent and heated at 120 C. Preparative TLC was carried out on plates coated with 0.5 mm of silica gel (MERCK); after development, the plates were sprayed with a 0.1% solution of berberin hydrochloride in ethanol. The compounds were observed under U.V. light (340 nm). Unless stated otherwise, CH_2Cl_2 was used as developing and eluting solvent. Gas liquid chromatography (GLC) was performed on a Packard model 427 equipped with a glass column (2 m x 3 mm) packed with 1% SE-30 and maintained at 270 C. Proton Magnetic Resonance (PMR) spectra were recorded at 250 MHz in $CDCl_3$ solution on a CAMECA spectrometer; the chemical shifts of signals are given in δ with Trimethylsilane (TMS) as internal standard. Mass spectra were done on an LKB 9000 mass spectrometer at an ionizing energy of 70 eV. The circular dichroism (C.D.) curves were measured in dioxane at 20 C on a Dichrograph III Jobin-Yvon instrument. The C.D. results are quoted as Δε (λ in nm), the concentration being referred to in mg/ml.

Cycloeucalanol (V)

I (430 mg) in cyclohexane (50 ml) was hydrogenated for 3 hr over platinum oxide (200 mg). After removal of the catalyst by filtration and evaporation of the solvent, the product was crystallized from chloroform-methanol to give V (410 mg, 95% yield) as needles m.p. 150° αD +50 C (c.0.8). I.R. νmax (KBr): 1040, 1000, 960, 895 cm^{-1}.

Cycloeucalanone (VII)

To V (410 mg) dissolved in a mixture of acetone (15 ml) and CH_2Cl_2 (4 ml), a solution of chromic acid (1.33 g CrO_3 + 1.15 ml H_2SO_4 + 3.85 ml H_2O) was added dropwise (O C, under N_2) with stirring and cooling till a brown color persisted (2.8 ml). After the solution was stirred at O C for 20 min, ethanol (2 ml) was added and the mixture evaporated, extracted with ether (50 ml x 3), washed with NaCl saturated solution (15 ml x 3), and dried (Na_2SO_4). After evaporation of the solvent, the residue was chromatographed on preparative TLC to yield (VII) (360 mg, 87.8% yield), m.p. 106°, α_D +48 C (c 0.8) C.D. Δε 0.88 (λ max 290) (c 0.25). I.R. νmax ($CHCl_3$): 1725, 1080, 960, 890, 875 cm^{-1}. MS = m/e 426 (M^+, 100%), 411 (28), 328 (9), 302 (16), 300 (22), 299 (93), 281 (10), 221 (10), 203 (14).

[1]Cycloeucalenol = 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol. (I); Obtusifoliol = 4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (II); 24-methylene cycloartanol = 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (III); 4β,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3β-ol (IV); Cycloeucalanol = 4α,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3β-ol (V); 4β,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3β-ol (VI); Cycloeucalanone = 4α,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3-one (VII); 4α,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholest-1-en-3-one (VIII); 4β,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3-one (IX); 4α,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3β-ol (X); 4α,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3-one (XI); 4β,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3-one (XII); 4,4,14α,(24ξ)-24-tetramethyl-9β,-19-cyclo-5α-cholestane-3β-ol (XIII); 4,4,14α,(24ξ)-24-tetramethyl-5α-cholest-8-en-3β-ol (XIV); 4α,14α,(24ξ)-24-trimethyl-5α-cholest-9(11)-en-3β-ol (XV); 4α,14α,(24ξ)-24-trimethyl-5α-cholest-7-en-3β-ol (XVI); 4β,14α,(24ξ)-26-trimethyl-5α-cholest-9(11)-en-3β-ol (XVII); 4β,14α,(24ξ)-24-trimethyl-5α-cholest-7-en-3β-ol (XVIII).

4α,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholest-1-en-3-one (VIII)

Bromide (17 μl) was added over about 20 min to VII (140 mg) dissolved in 14 ml of buffer solution (0.2 g of sodium acetate in 160 ml of glacial acetic acid and 4 ml of CCl_4). When the mixture was no longer colored, $CHCl_3$ (5 ml) was added to the mixture, which was stirred for about 15 min. The crude mixture was successively washed with water, aqueous $NaHCO_3$, and water again. After evaporation of the solvent, the residue (120 mg) was heated under reflux with LiCl (400 mg) in dimethylformamide (DMF) (10 ml) under N_2 for 3 hr. The usual workup gave VII, IX, and VIII. VIII: U.V. λmax 265nm (ε7900), I.R. (K Br) λmax 1680, 1670, 1610, 935, 920, 865, 820 cm^{-1}. M.S. = m/e 424 (M^+, 100%), 409 (5), 381 (5), 353 (25), 340 (4), 326 (21), 297 (50), 245 (13), 203 (13), 201 (13), 189 (10), 187 (11), 175 (36). P.M.R. ($CDCl_3$). δ: 0.54 (1H, one of AB doublet, J=4 Hz, cyclopropane) 6.9 and 6.0 (2H, AB doublets, J=10 Hz, two olefinic protons), 0.860 (d, 3H, J=6.5Hz, C-21-methyl), 0.908 (3H, s, C-14-methyl), 0.989 (d, 3H, J=6.3Hz, C-4-methyl, 1.002 (3H, s, C-18-methyl).

4β,14α,(14ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3-one (IX)

VII (300 mg) was heated at reflux under N_2 for 3 hr with LiCl (0.65 g) in DMF (15 ml). The reaction mixture was evaporated under reduced pressure Analysis by TLC indicated the presence of two components (R_f0.53 and 0.43), the less polar of which corresponded to unreacted VII (240 mg, 80%) and to IX (22,5 mg, 7.5%) m.p. 128-130C ($CHCl_3$-MeOH), C.D. Δε 0.39 (λmax 300 nm) (c 1.8). MS = m/e 426 (M^+, 76%), 411 (24), 328 (14.5) 302, (18.5), 300 (21), 299 (100), 281 (5.6), 221 (6), 203 (7.7). I.R. (KBr) νmax 1725, 1115, 1025, 960, 895 cm^{-1}. VII (20 mg) treated under reflux with LiBr (260 mg) in DMF (3 ml) gave unreacted VII (16 mg), 80%) and IX (1.5 mg, 7.5%).

4β,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3β-ol (IV)

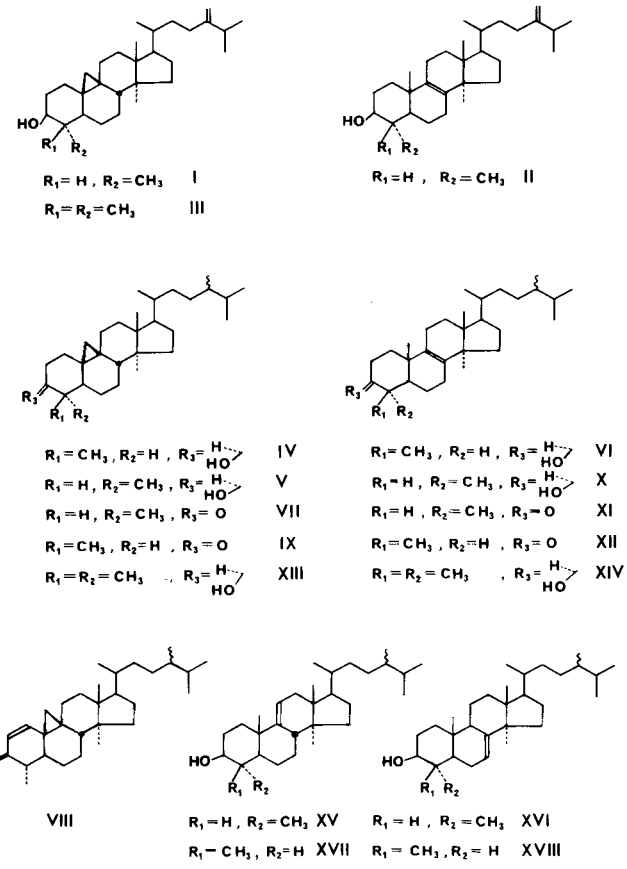
$LiAlH_4$ (20 mg) was added to a solution of VII (20 mg) in THF (2 ml). After this solution was stirred for 45 min at room temperature, a 20% aqueous solution of NaH_2PO_4 (1 ml) was added to decompose the unreacted hydride, and the mixture extracted with diethyl ether (5 ml x 3), dried, and evaporated under reduced pressure. The residue was purified by preparative TLC to yield IV (15 mg, 75%), m.p. 129-130C ($CHCl_3$-MeOH), R_f (CH_2Cl_2) 0.180, I.R. (KBr) νmax 1110, 1035, 1020, 990, 960, 890 cm^{-1}.

4α,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3β-ol (X)

Obtusifoliol (II) (22 mg) in cyclohexane (5 ml) was hydrogenated for 3 hr over PtO_2 (12 mg). After the usual workup, X (20 mg) was obtained, m.p. 124C ($CHCl_3$-MeOH), α_D + 68 C (c 1.88), R_f (CH_2Cl_2) 0.240.

4α,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3-one (XI)

A solution of Jones reagent (0.15 ml) was added dropwise at 0C under N_2 to X (20 mg) dissolved in acetone (15 ml)-CH_2Cl_2 (0.4 ml). The reaction mixture was worked up in the usual manner, and the product was purified by preparative TLC and crystallization from MeOH-$CHCl_3$ to yield XI (12 mg), m.p. 105C, α_D + 40C (c 1.5), MS: m/e 426 (M^+,42%), 412 (36), 411 (100), 395 (25), 328 (4), 302 (4), 307 (6), 287 (9), 279 (15), 259 (6), 245 (11), 233 (11), 227 (7), 215 (7), 213 (5), 201 (8).



EQUATION I

4β,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3β-ol (VI)

XI (12 mg) was treated with LiCl (130 mg) in DMF (3 ml) and heated at reflux under N_2 for 3 hr. After the usual workup, the crude residue was purified by preparative TLC to give the starting unreacted ketone (XI) (10 mg) and a more polar compound (XII) (0,5 mg, 4%) R_f (CH_2Cl_2) 0.45. MS practically identical to XI. The isomerization procedure was repeated 4 times utilizing recovered XI. XII (1.5 mg), dissolved in anhydrous tetrahydrofuran (THF) (1 ml), was reduced with $LiAlH_4$ (3 mg) to give VI (1.2 mg).

Miscellaneous Compounds

4,4,14α-(24ξ)-24-tetramethyl-9β,19-cyclo-5α-cholestane-3β-ol (XIII) was prepared by hydrogenation of 24-methylene cycloartanol (II) over PtO_2. 4,14α,(24ξ)-24-trimethyl-5α-cholest-9(11)-en-3β-ol (XV) and 4,14α,(24ξ)-24-trimethyl-5α-cholest-7-en-3β-ol (XVI) were prepared from V as described previously (8). Cycloartenol was the kind gift of Dr. A.S. Narula (Baroda, India). Cycloeucalenol (I) was extracted from tallow wood as described previously (7). Tallow wood was kindly supplied by Prof. R.A. Massey-Westropp (Adelaide, Australia). Obtusifoliol (II) was purified from a crude extract of latex from *Euphorbia obtusifolia*, kindly supplied by Prof. A. Gonzales-Gonzales (Las Palmas, Las Canarias, Spain). 24-methylene cycloartanol (III) was kindly supplied by Dr. Itoh (Tokyo, Japan).

Preparative of Subcellular Fractions and Enzymatic Assays

Experimental details have been given to previous articles (7,8): Corn seeds (*Zea mays*, va. INRA 258) were soaked for 5 hr in H_2O before being planted in moist vermiculite. They were grown for 60 hr at 25C. The embryos (70 g)

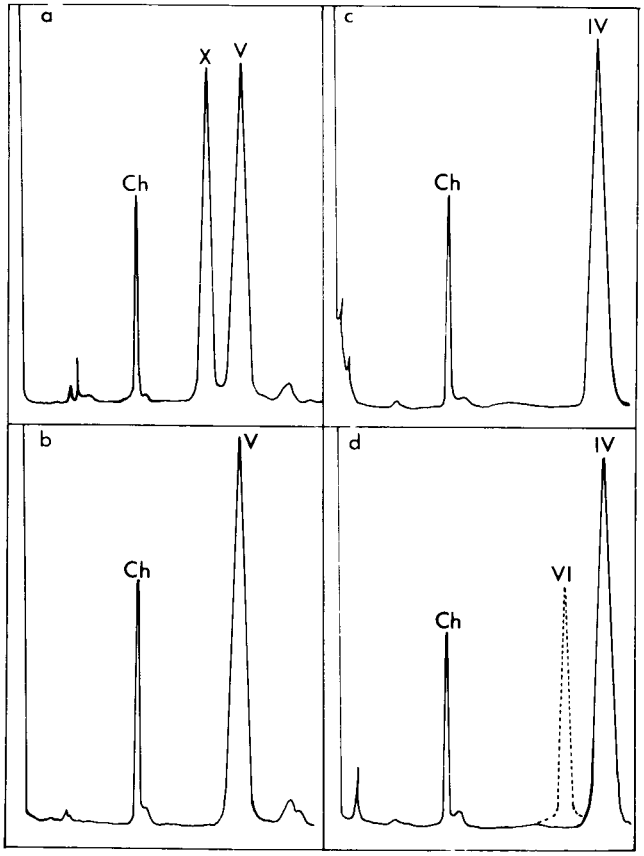


FIG. 1. Gas liquid chromatograms corresponding to the analysis of the 4α-methyl sterol fraction (a,b) and the 4β-methyl sterol fraction (c,d) originating from a 4 hr incubation of V (a,b) and IV (c,d) with microsomes from maize embryos b and d: boiled microsomes (control). Ch = cholesterol. The dotted lines in d represent the position occupied by VI when IV and VI are coinjected. Roman numbers represent substrates and products (see nomenclature).

were ground in a mortar at 0C with 3 vol. of medium containing 0.1 M Tris-HCl, 0.3% BSA, 10 mM mercaptoethanol, 0.5 M Sucrose, 4 mM $MgCl_2$, final pH 7.5. The homogenate was squeezed through two layers of cheescloth and centrifuged at 2000 g for 6 min. The supernatant was centrifuged at 105 000 g for 1 hr. The soluble supernatant was removed, and the microsomal pellets were suspended in 15 ml of medium containing 0.1 M phosphate buffer pH 8.3,5 mM mercaptoethanol and 2 mM $MgCl_2$, and dispersed in a Potter-Elvehjem homogenizer.

The dispersed microsomal pellet (3.5 ml) was incubated in the presence of the substrate (100 μM) dissolved in 0.6% Tween-80 in H_2O (0.5 ml) for various periods of time (from 2 to 12 hr) at 31C. The reaction was terminated by the addition of 20% KOH in EtOH (1 vol.).

Analytical Procedures

The incubation mixture was extracted 3 times with petrol (50 ml). Combined extracts were dried over Na_2SO_4, evaporated in vacuo, and separated by TLC with CH_2Cl_2 as the solvent (2 runs). The bands of 4α-methyl sterols (R_f 0.24), 4β-methyl sterols (R_f 0.18), and 4,4-dimethyl sterols (R_f0.30), depending on whether 4α-methyl sterols (like V), 4β-methyl sterols (IV), or 4,4-dimethyl sterols (XIII) respectively, had been used as substrates, were scraped off and eluted. Each band was analyzed by GC-MS. Acetylation was performed as described previously, and the acetates were chromatographed on argentated thin layer places allowing separation of isomeric products possibly arising from IV, V or XIII.

Detection of the enzymatically formed products was performed by GLC on the free alcohols. The carrier gas was N_2 (30 ml/min). A glass column packed with 1% SE-30 was used at 250C. Quantitative determinations were made by measuring the relative peak areas of the substrate and the product. These areas were compared to that of an internal standard of cholesterol and to that of a known amount of substrate originating from a control incubation of boiled microsomes.

RESULTS

Preparation of the 4β-Methyl Steroids

The general procedure for synthesizing the 4β-methyl steroids consisted of the catalytic hydrogenation of the 4-methyl-Δ4-steroid derivative by normal cis addition of hydrogen from the α-side of the molecule (9,10). Dihydrocyclobuxine D, a 4β-methyl-9β,19-cyclosteroid derivative, was also prepared by catalytic hydrogenation of cyclobuxine D (6). Therefore, we planned to synthesize (IV) from 4,14α,(24ξ)-24-trimethyl-9β,19-cyclo-cholest-4-ene-3-one by catalytic reduction. Hydrogenation of cycloeucalenol (I) over PtO_2 afforded cycloeucalanol (V), which by oxidation with Jones's reagent at 0 gave cycloeucalanone (VII). VII was brominated under controlled conditions (11) to give a crude mixture which, without further purification, was treated with LiCl in dimethylformamide, to yield VIII, the saturated started ketone, and, surprisingly, 4β,14α,(24ξ)-24-trimethyl-9β,19-cyclo-cholestane-3-one (IX) in small yield, which probably derived from VII present in the crude bromination mixture by isomerization. In order to verify this assumption, we heated VII under reflux with LiCl in DMF to obtain a mixture of cycloeucalanone (VII, 80% yield) and its 4β-epimer (IX, 7.5% yield). LiCl could be replaced by LiBr, whereas collidine, $LiCO_3$, or DMF alone did not produce isomerization of VII to IX. Reduction of IX with $LiAlH_4$ in THF gave 4β,14α,(24ξ)-24-trimethyl-9β,19-cyclo-cholestane-3β-ol (IV).

The same synthetical approach was also used for the preparation of 4β,14α,(24ξ)-24-trimethyl-5α-cholest-8-en-3β-ol (VI). Obtusifoliol (II) was hydrogenated in the presence of PtO_2 in cyclohexane to give X, which, after Jones oxidation, gave XI. Treatment of XI with LiCl in DMF afforded the starting ketone (XI) and the 4β-methyl epimer (XII), which, after reduction with $LiAlH_4$, gave VI.

Incubation of the 4β-Methyl Sterols

4β,14α,(24ξ)-24-trimethyl-9β,19-cyclo-5α-cholestane-3β-ol (IV) and its 4α epimer, cycloeucalanol (V), were incubated with microsomes from maize embryos prepared as described previously (8). Incubations were performed for 4 hr at 30 C in the presence of two controls consisting of boiled microsomes with IV or V added. The two incubations and the two controls were extracted as described in the experimental section, the extracts were chromatographed on TLC, and the 4-methyl sterol fractions were recovered and submitted to GLC. Gas liquid chromatograms corresponding to the two incubations and the two controls are shown in Figure 1. The controls allowed us to take into account the presence of compounds present in maize microsomes and having the same RRT as the expected formed compounds, i.e., the 4α- and 4β-methyl-Δ-8-epimers, respectively X and IV. Whereas cycloeucalanol (V) was converted into a compound having the same RRT and mass spectrum as authentic 4α,14α,(24ξ)-24-trimethyl-cholest-8-en-3β-ol (X), the 4β-methyl epimer (IV) or cycloeucalanol was not transformed into a compound with an RRT identical to that of the expected 4β,14α,(24ξ)-24-trimethyl-cholest-8-en-3β-ol (VI) (Figure 1, a and c). As shown previously, HCl

opening of the cyclopropane ring of 9β,19-cyclosteroids gave a mixture of Δ-9(11), Δ-8, and Δ-7 isomers; in contrast, the enzymic opening of the cyclopropane ring of cycloeucalenol (I) yielded only obtusifoliol (II), a Δ-8 compound (8). It could be suggested that this selectivity could be lost when a 4β-methyl,9β,19-cyclosteroid such as (IV) was used as substrate in the enzymic reaction and that Δ-7 or Δ-9(11) compounds could be formed, as in the case of HCl treatment. Whereas 9β,19-cyclosteroids were shown to be unambiguously separated by GLC from their Δ-8 isomers, they were poorly separated from Δ-7 and Δ-9(11) isomers (Table I) (8,12). To achieve better separation, the crude 4α- and 4β-methyl sterol fractions were acetylated, and the acetates were separated using preparative argentation thin layer chromatography. These conditions allowed the Δ-9(11) to be separated easily from the unreacted substrate and the Δ-7 and the Δ-8 isomers (Table I). Total resolution was achieved by epoxidating the acetates of the substrate, and of the Δ-7 and Δ-8 isomers as described previously (7,8). As no trace of the Δ-7 and Δ-9(11) isomers (XVII and XVIII) were detectable in these conditions, it could be concluded that the cyclopropane ring of the 4β-methyl-9β,19-cyclosteroid was not opened in conditions in which that of the 4α-methyl epimer was opened with a yield higher than 40%.

Finally 4,4,14α,(24ξ)-24-tetramethyl-9β,19-cyclo-5α-cholestane-3β-ol (XIII) was incubated in the same conditions as before. As previously demonstrated, in the case of 24-methylene cycloartanol (III) and other 4,4-dimethyl-9β,-19-cyclosteroids (7), XIII was not transformed either into 4,4,14α,(14ξ)-24-tetramethyl-5α-cholest-8-en-3β-ol (XIV) or into the Δ-9(11) and the Δ-7 isomers of XIV.

TABLE I

R_f (TLC) and Relative Retention Times (GLC) of the 4α- and 4β-methyl Sterols Studied in the Present Work

		R_f[a]	R_f[b]	RRT[c]
4α-methyl sterols	IV	0.24	0.50	1.72
	VI Δ-8	0.24	0.50	1.49
	XV Δ-9(11)	0.24	0.30	1.65
	XVI Δ-7	0.24	0.45	1.73
4β-methyl sterols	V	0.18	0.50	1.86
	X Δ-8	0.18	0.50	1.70
	XVII Δ-9(11)	0.18	0.30	
	XVIII Δ-7	0.18	0.45	
4α-methyl ketones	VII	0.53	---	1.73
	XI	0.53	---	---
4β-methyl ketones	IX	0.43	---	1.78[
	XII	0.43	---	---

[a](CH_2Cl_2x2).
[b]Acetates, argentation TLC (cyclohexane/toluene, 60:40, v/v).
[c]Retention time for cholesterol is taken as 1.00. (1% SE-30 column).

DISCUSSION

Synthesis of the 4β-Methyl Sterols

The formation of 4β,14α,(24ξ)-24-trimethyl-9β,19-cyclocholestane-3-one (IX) and of 4β,14α,(24ξ)-24-trimethylcholest-8-en-3-one (XII) by isomerization of the corresponding 4α-methyl epimers (VII) and (XI) in the presence of LiCl and DMF represented a unique and unexpected result. Generally the 4β-methyl (axial) derivatives are readily isomerized to the more stable 4α-methyl (equatorial) isomers by brief reflux in acid ethanol (9,10), whereas attempts of epimerization under alkaline conditions have been unsuccessful (13). LiCl or LiBr in boiling DMF was shown to be especially effective in the dehydrobromination of the steroidal α-bromo ketones (14). This reaction seems to proceed through a base-catalyzed enolization mechanism (15). It has been proposed that Br^- or Cl^- acts as a base in removing the β-proton to the carbonyl group, as the reactivity of halide ions is increased by the use of the aprotic, although highly polar, DMF (16,17). In the case of epimerization of cycloeucalanone (VII), the axial C-4 hydrogen could be abstracted by the halide ion in DMF, and the Li^+ cathion may act as a Lewis acid in stabilizing the anionic transition state involved during the enolization of the carbonyl group. The failure of collidine, DMF, and $LiCO_3$-DMF to isomerize the 4α-methyl sterols into the 4β-epimers seems to support the above hypothetical mechanisms.

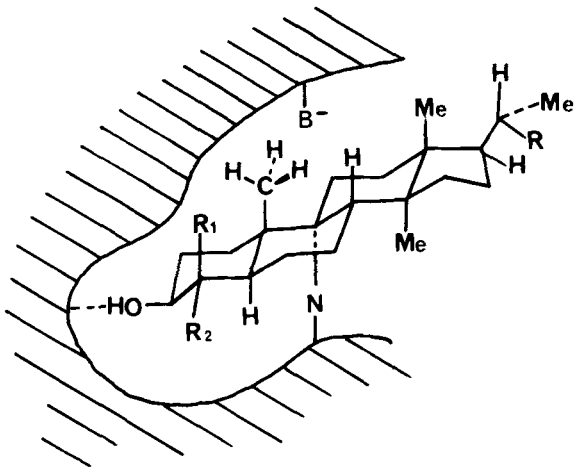
There are striking differences in the chromatographic and PMR properties of the isomeric 4α- and 4β-methyl derivatives. According to published data (10,18), the axially-substituted 4β-methyl steroids are more polar on TLC and GLC than are the epimeric 4α-methyl derivatives. Therefore, the 4β-methyl and the 4α-methyl sterols considered in this work (IV, VI and V, X) can be easily distinguished from one another by simple TLC and GLC analysis (Table

TABLE II

Chemical Shifts (δ,ppm) of Proton Signals of IV, V, VI, VII, IX, and X in PMR Spectroscopy (250 MHz)

	C-13	C-14	C-21	C-26	C-27	C-28	C-10		C-4	C-3-H
V	0.966 s	0.890 s	---	0.849 d J=6.75[a]	0.806 d J=6.0	0.780 d J=6.75	0.143 dd	0.384	0.979 d J=6	3.224 m
IV	0.959	0.901	0.889 d J=6.5	0.854 d J=6.75	0.805 d J=7.0	0.778 d J=6.5	0.313 dd	0.582	0.884 d J=8.5	3.740 m
VII	1.002 s	0.908 s	0.890 d J=6.5	0.860 d J=7.0	0.811 d J=6.5	0.785 d J=6.5	0.394 dd	0.617	0.990 d J=6.5	---
IX	0.993 s	0.918 s	0.904 d J=6.5	0.858 d J=7.0	0.809 d J=6.5	0.782 d J=6.75	0.216 d J=4 0.563 d J=6.5	0.301 s 0.606 s	1.138 d J=7	---
X	0.706 s	0.883 s	0.886 d J=6.5	0.854 d J=6.0	0.806 d J=6.0	0.780 d J=6.0	0.970 s		0.997 d J=6.5	3.116 m
VI	0.687 s	0.879 s	0.886 d J=6.5	0.853 d J=7	0.805 d J=6.8	0.778 d J=6.5	0.963 s		0.878 d J=8	

[a]Coupling constants in Hz.



$R_1 = CH_3$, $R_2 = H$ XX

$R_1 = H$, $R_2 = CH_3$ XIX

SCHEME I

I). The two epimeric pairs of products can also be distinguished by their PMR spectra (Table II). The axial C-3α-H of (IV) is shifted 0.53 ppm downfield as compared to that of the 4α-methyl epimer (V). In addition, the C-4β-methyl signals of (IV) and (VI) resonate at higher fields than those of the corresponding C-4α methyl isomers (V) and (X). Moreover, the coupling constant of the C-4β methyl group is markedly larger (J = 8-8.5 Hz) than that observed for the 4α-methyl group (J = 6.0-6.5 Hz). Comparison of the spectra of 4-monomethyl-3β-hydroxy steroids with their 3-keto derivatives also revealed that the C-4β methyl group is strongly deshielded (0.25 ppm) by changing the C-3 functional group from hydroxyl to carbonyl, whereas the C-4α methyl group is scarcely affected (0.04 ppm). The large deshielding of the C-4β methyl group is believed to be due to magnetic anisotropy effects of the C-3 carbonyl group on the methyl, as it lies outside the conical region, extending above the plane of the trigonal carbon atom of the C=O bond (19). Comparison of the PMR spectra of 4β,14α,(24ξ)-24-trimethyl-9β,10-cyclo-cholestane-3β-ol (IV) and cycloeucalanol (V) showed that the doublets of cyclopropyl methylene protons of the former were shifted downfield by 0.16 and 0.18 ppm. Probably the intramolecular interaction of the C-4-methyl group in an axial arrangement exerts a deshielding effect on the two cyclopropyl methylene protons (20). All these results are perfectly in agreement with those published for 4α- and 4β-methyl steroids in the cholestane series (10,18-20) and for natural and synthetic Buxus alcaloids (5). The circular dicroism curves of VII and IX showed a maximum at 290 ($\Delta\epsilon$=0.88) and 300 ($\Delta\epsilon$=0.39) respectively. As expected, VII exhibited a positive Cotton effect, whereas the axially oriented methyl grou] in the 9β,10-cyclosteroid skeleton of IX produced a marked reduction in amplitude without, however, inverting the sign of the single Cotton-effect curve (21). The mass spectra of the isomeric pairs (V, IV; VII,IX; X,VI and XI, XII) are essentially identical although there may be differences in the relative intensities of some ions.

Results of Incubation

Results of the incubation of the isomers of 4-methyl-cyclopropyl sterols gave the following information. First, the 4α-epimer (V) was converted to dihydro-obtusifoliol (X) by microsomes from maize embryos. The yield of this conversion was reproducibly about 40% for a 4 hr incubation, but reached 80% and even more for a 12 hr incubation (results not shown here). The reaction stopped when the substrate was nearly completely exhausted. Second, the 4β-epimer (IV) was not converted to the expected 4β,14α(14ξ)-24-trimethyl-5α-cholest-8-en-3β-ol (VI). The sensitivity of the method, used to detect any formed IV, excluded any conversion of IV higher than 2%. Third, XIII, a 4,4-dimethyl-cyclopropyl sterol, was not transformed into the expected (24ξ)-24-methyl-24-dihydro-lanosterol (XIV), in total agreement with results obtained previously following incubation of cycloartenol and 24-methylene-cycloartanol (II) (7,8).

Enzymatic isomerization of 9β,19-cyclopropyl sterols to Δ-8-sterols involves two events: a. protonation and then opening of the cyclopropane ring, leading to the formation of a carbonium ion at C-9; b. elimination of a proton at C-8. These two events are probably not concerted, since the 9β-C19 and 8β-H bonds are cis to each other; because of that, an intermediate involving an ion pair between the carbonium ion and a suitable nucleophilic group of the enzyme is generally postulated (22) (Scheme 1). From previously obtained results (8), it has been proposed that during incubation of a 4,4-dimethyl cyclopropyl sterol like XIII, it is the first process, i.e., the opening of the cyclopropane ring, which is inhibited. To explain this inhibition it was suggested that the presence of two methyl groups at C-4 in place of one 4α-methyl hinders the action of the enzyme. The fact that IV, a 4β-methyl-cyclopropyl sterol, was not transformed by the enzyme clearly indicates that not only the number but also the stereochemistry of the C-4 methyl controls the enzymic reaction, and that probably the 4β-methyl group could either hinder the binding of IV (or XIII) on the enzyme or hinder the access of the substrate to a specific site on the enzyme (the H^+ donor, for example). In addition, the opening of the cyclopropane ring of IV (or XIII) involves unfavorable 4β-methyl, 10-methyl interactions, i.e., high transition-state energy in intermediate XX which does not exist in intermediate XIX. Therefore, from a thermodynamic and kinetic point of view, one would expect the opening of the cyclopropane ring to be more favored in the case of V than in the case of IV.

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