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Metabolism of 27-Nor-24,25-dihydrolanosterol and 23,24,25,26,27-Pentanordihydrolanosterol by Rat Liver Homogenate Preparations[1)]

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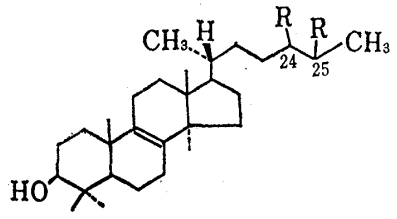
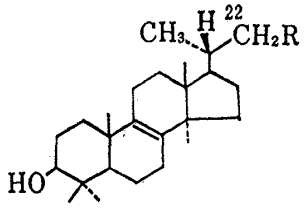
The metabolism of 27-nor-24,25-dihydrolanosterol (**1**) which is an effective inhibitor of cholesterol biosynthesis from lanosterol was studied using the unlabeled and 24,25-tritiated compounds. **1** was transformed into the cholesterol analog, 27-norcholesterol, to the extent of 6.5% by incubation with rat liver homogenate under conditions such that lanosterol in the control experiment was converted to cholesterol to the extent of 24.8%. The structures of two other metabolites (**9** and **11**) were determined. On the other hand, 23,24,25,26,27-pentanordihydrolanosterol (**2**), which is also an inhibitor, was not transformed into the corresponding cholesterol analog, suggesting the importance of the side chain structure of lanosterol in cholesterol biosynthesis.

Keywords——[24,25-^{3}H]-27-nor-24,25-dihydrolanosterol; [22-^{3}H]-23,24,25,26,27-pentanordihydrolanosterol; rat liver homogenate preparations; inhibitor; cholesterol biosynthesis; metabolite; 27-norcholesterol

We have previously shown[1)] that lanosterol analogs with both a side chain longer than the hexanor analog and the normal configuration at C-20 are potent inhibitors of cholesterol biosynthesis from lanosterol. This finding prompted us to determine whether these lanosterol analogs can be metabolized as substrates. Previously Van Tamelen *et al.*[2)] showed that trisnorsqualene is converted to trisnorcholesterol by incubation with rat liver preparations, suggesting that trisnorlanosterol may be converted to the trisnorcholestane series. On the other hand, it is of interest to test lanosterol analogs as substrates of cholesterol biosynthesis, since studies on analogs with modified side chains will give information on the mode of interaction between this enzyme system and its substrate. For this purpose we prepared the tritium-labeled 27-nor-24,25-dihydrolanosterol (**1b**) and 23,24,25,26,27-pentanordihydrolanosterol (**2b**), and examined their metabolism in preparations of rat liver *in vitro*.



1a : R=H
1b : R=^{3}H



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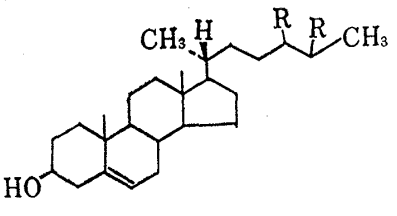
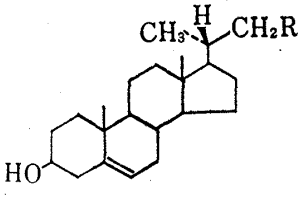


3a : R=H
3b : R=^{3}H



4a : R=H
4b : R=^{3}H

Chart 1

The incubation of [24,25-^{3}H]-27-nor-24,25-dihydrolanosterol (**1b**) with rat hepatic subcellular preparations (S-10)[3] afforded the corresponding demethylated sterol, [24,25-^{3}H]-27-norcholesterol (**3b**), but that of [22-^{3}H]-23,24,25,26,27-pentanordihydrolanosterol (**2b**) resulted in the recovery of the starting material without transformation into tritium-labeled 23,24,25,-26,27-pentanorcholesterol (**4b**).

Materials and Methods

Analyses of Lanosterol and Cholesterol Derivatives——Proton nuclear magnetic resonance (PMR) spectra and mass spectra (MS) were recorded as described previously.[4]

Synthesis of [24,25-^{3}H]-27-Nor-24,25-dihydrolanosterol (1b)——**1b** was synthesized as described previously[4] with some modifications. 3β-Acetoxy-27-norlanosta-8,24-diene (20 mg) in MeOH (25 ml) was tritiated under a tritium atmosphere (5 Ci) in the presence of 5% Pd-C (40 mg) at the Radiochemical Centre, Amersham, England. Aliquots (50 mCi) of the filtered solution were mixed with the carrier (3β-acetoxy-27-norlanost-8-ene, 45 mg), and the mixture was hydrolyzed with 10% KOH–MeOH. The product was extracted with CH_2Cl_2 after dilution with water and the extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was recrystallized from MeOH to a constant specific activity (20 mg, 459 mCi/mmol).

Synthesis of [22-^{3}H]-23,24,25,26,27-Pentanordihydrolanosterol (2b)——**2b** was synthesized as described previously[4] with some modifications. [^{3}H]-$LiAlH_4$ (5 mCi) and $LiAlH_4$ (50 mg) were added to a solution of 3β-acetoxy-22-chloro-23,24,25,26,27-pentanorlanost-8-ene (7 mg) in anhydrous dioxane and the mixture was refluxed for 20 h. The product was extracted with CH_2Cl_2 after dilution with water and the extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was chromatographed on silica gel (5 g). Elution with benzene gave the starting material (3.7 mg). Further elution with benzene–CH_2Cl_2 (9: 1) gave a solid which was recrystallized from MeOH to give colorless needles of **2b** (1.5 mg, 0.10 mCi/mmol). **2b** exhibited on both thin layer chromatography (TLC) and gas liquid chromatography (GLC) the same chromatographic mobilities as the unlabeled authentic sample (**2a**).[4]

Synthesis of 27-Norcholesterol (3a)——An *n*-butyl lithium solution in hexane (14%, 1.2 ml) was added to a suspension of *n*-butyl triphenylphosphonium bromide (1.2 g) in anhydrous benzene (20 ml). The mixture was stirred at room temperature for 10 min, then a solution of 3β-acetoxybisnorchol-5-en-22-al[5] (0.5 g) in anhydrous benzene (10 ml) was added, and the mixture was further stirred at room temperature for 30 h. After extraction with benzene, the residue was chromatographed on silica gel (30 g). Elution with benzene gave a solid (0.31 g) which was recrystallized from MeOH to give colorless needles of 22-dehydro-27-norcholesterol (a mixture of *trans* and *cis* isomers, 1: 3) (**5**), mp 121—122°C. *Anal.* Calcd for $C_{26}H_{42}O$: C, 84.26; H, 11.42. Found: C, 84.64; H, 11.78. MS *m/e*: 370 (M^+), 355, 352. PMR δ (ppm): 0.70 and 0.72 (s and s, 18-CH_3 of 22-*trans* and *cis*, 1: 3), 0.94 and 0.96 (d and d, 21-CH_3 of 22-*trans* and *cis*, 1: 3), 1.00 (s, 19-CH_3), 3.50 (m, 3α-H), 5.34 (dd, 6-H), 5.10—5.28 (m, 22 and 23-H).

5 (100 mg) in MeOH (80 ml) was hydrogenated in the presence of 5% Pd-C (50 mg) at room temperature for 1 h. After removal of the catalyst by filtration, the filtrate was evaporated to dryness. The residue was recrystallized from MeOH to give colorless needles of **3a** (85 mg), mp 131—134°C. *Anal.* Calcd for $C_{26}H_{44}O$: C, 83.80; H, 11.90. Found: C, 83.92; H, 11.92. MS *m/e*: 372 (M^+, base peak), 357, 354, 339. PMR δ (ppm): 0.68 (3H, s, 18-CH_3), 0.91 (3H, d, 21-CH_3, J=6 Hz), 1.00 (3H, s, 19-CH_3), 3.50 (1H, m, 3α-H), 5.32 (1H, dd, 6-H).

23,24,25,26,27-Pentanorcholesterol (3β-Hydroxybisnorchol-5-ene) (4a)——**4a** was synthesized as described previously[5] (mp 137—138°C).

Synthesis of 4α-Methylcholest-7-en-3-one (6)——**6** was synthesized by the method of Neiderhiser *et al.*[6] Recrystallization from MeOH gave colorless needles of mp 137—138°C (lit.,[6] 136—137°C). MS *m/e*: 398 (M^+, base peak), 383, 285, 259, 243. PMR δ (ppm): 0.57 (3H, s, 18-CH_3), 0.86 (6H, d, J=6.5 Hz, 26 and 27-CH_3), 0.96 (3H, s, 19-CH_3), 1.03 (3H, d, J=5 Hz, 4α-CH_3), 5.20 (1H, bd, 7-H).

Syntheses of 4α-Methylcholest-8(14)-en-3-one (7) and 4α-Methylcholest-14-en-3-one (8)——Dry HCl gas was passed through a solution of **6** (50 mg) in $CHCl_3$ (25 ml) at room temperature for 15 min. The reaction mixture was repeatedly washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was chromatographed on 10% $AgNO_3$-impregnated silica gel (10 g). Elution with benzene gave a solid (23 mg), which was recrystallized from MeOH to give colorless needles of **7**, mp 97—98°C. *Anal.* Calcd for $C_{28}H_{46}O$: C, 84.35; H, 11.63. Found: C, 84.39; H, 11.55. MS *m/e*: 398 (M^+, base peak), 383, 285, 259, 243. PMR δ (ppm): 0.86 (6H, d, J=6.5 Hz, 26 and 27-CH_3), 0.89 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 1.02 (3H, d, J=5 Hz, 4α-CH_3). Further elution with benzene gave a solid (13 mg), which was recrystallized from MeOH to give colorless needles of **8**, mp 104—105°C. *Anal.* Calcd for $C_{28}H_{46}O$: C, 84.35; H, 11.63. Found: C, 84.72; H, 11.45. MS *m/e*: 398 (M^+), 383, 285 (base peak). PMR δ (ppm): 0.86 (6H, d, J=6.5 Hz, 26 and 27-CH_3), 0.90 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 1.05 (3H, d, J=5 Hz, 4α-CH_3), 5.14 (1H, m, 15-H).

Synthesis of 27-Norcholest-5-en-3-one (9)——Jones reagent (0.2 ml) was added to a solution of 27-norcholesterol (0.22 g) in acetone (20 ml) and the reaction mixture was stirred at room temperature for 15 min. After removal of the chromium salts by filtration, ice-water was added to the reaction mixture. The whole was extracted with CH_2Cl_2, washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was chromatographed on silica gel (10 g). Elution with benzene gave a solid (0.11 g) which was recrystallized from MeOH to give colorless needles of **9**, mp 105—107°C. *Anal.* Calcd for $C_{26}H_{42}O$: C, 84.26; H, 11.42. Found: C, 84.12; H, 11.60. MS *m/e*: 370 (M^+), 355, 124 (base peak). PMR δ (ppm): 0.72 (3H, s, 18-CH_3), 0.92 (3H, d, J=6.5 Hz, 21-CH_3), 1.18 (3H, s, 19-CH_3), 5.32 (1H, bd, 6-H).

Preparations of Rat Hepatic Subcellular Fractions and Incubation Procedure——Hepatic subcellular 10000×**g** supernatant fractions (S-10) were prepared as described previously.[1] Incubation procedures were the same as described previously.[1]

Analyses of Incubation Products of [22-^{3}H]-23,24,25,26,27-Pentanordihydrolanosterol (2b) and [24,25-^{3}H]-27-Nor-24,25-dihydrolanosterol (1b)——The 4,4-demethyl sterol fraction obtained from the incubation of **2b** was diluted with unlabeled 23,24,25,26,27-pentanorcholesterol (**4a**), recrystallized from MeOH, and counted in a liquid-scintillation spectrometer (Aloka LSC-502). The products from the reaction of **1b** were analyzed by radioautography, and the band containing 4,4-demethyl sterols was eluted from the TLC plate of silica gel with MeOH (100 ml). The radioactive material was cocrystallized with unlabeled 27-norcholesterol (**3a**). The band containing 4,4-dimethyl sterols (*i.e.* lanosterol fraction) was eluted from the silica gel with MeOH (100 ml). The radioactive material was cocrystallized with unlabeled 27-nor-24,25-dihydrolanosterol to a constant specific radioactivity.

Incubation of Unlabeled 27-Nor-24,25-dihydrolanosterol (1a)——**1a** (40 μg) dispersed with 3 mg of Tween 80 was incubated with S-10 (4 ml) containing cofactors at 37°C for 3 h in five flasks and worked up as described in the case of tritium-labeled 27-nor-24,25-dihydrolanosterol (**1b**). The combined cholesterol fractions, 4,4-dimethyl sterol fractions and sterone fractions were analyzed by gas chromatography–mass spectrometry (GC–MS) (JEOL JMS D-100 machine).

Results and Discussion

To investigate the metabolic activity of rat liver homogenate, [22-^{3}H]-23,24,25,26,27-pentanordihydrolanosterol was incubated with S-10, and after TLC of the products (which is capable of separating 4,4-demethyl sterol, 4-monomethyl sterol, 4,4-dimethyl sterol and sterone),[7,8] a portion of the 4,4-demethyl sterol fraction was cocrystallized with 23,24,25,26,-27-pentanorcholesterol (**4a**). However, radioactivity indicative of the formation of **4a** was not detected in the sample. The 4,4-dimethyl sterol fraction was cocrystallized with 23,24,-25,26,27-pentanordihydrolanosterol (**2a**), and it was found that 87.5% of the starting material

Table I. Metabolites of [24-^{3}H]-Lanosterol, [24-^{3}H]-24,25-Dihydrolanosterol, [24,25-^{3}H]-27-Nor-24,25-dihydrolanosterol, and [22-^{3}H]-23,24,25,26,27-Pentanordihydrolanosterol

Substrate (Amount incubated)	Incorporation of radioactivity (%)			Recovered substrate (%)
	Cholesterol analog	4-Monomethyl sterols	Sterones	
[24-^{3}H]-Lanosterol (9.06×10^4 dpm, 40 μg)	24.8	12.6	9.8	25.3
[24-^{3}H]-24,25-Dihydrolanosterol (6.31×10^4 dpm, 40 μg)	23.4	13.1	9.6	25.8
[24,25-^{3}H]-27-Nor-24,25-dihydrolanosterol (9.85×10^7 dpm, 40 μg)	6.5	8.5	18.6	42.6
[22-^{3}H]-23,24,25,26,27-Pentanordihydrolanosterol (2.51×10^4 dpm, 40 μg)	0	0	0	87.5

Incubations were done at 37°C for 3 h with S-10 fraction (4 ml, 18.0 mg protein/ml) of rat liver homogenate in the presence of 30 mM nicotinamide, 10 mM GSH, 1 mM EDTA, 2 mM $NADP^+$, 0.8 mM NAD^+, 1.3 mM NADH, 12 mM glucose-6-phosphate, 1.3 mM ATP, 4 mM $MgCl_2$, 1 unit of glucose-6-phosphate dehydrogenase and 0.1 M potassium phosphate buffer (pH 7.4) in a total volume of 5.0 ml. The incubations were started by the addition of the substrate emulsion in potassium phosphate buffer, pH 7.4 (0.1 ml). On TLC, bands were identified by spraying authentic sterols spotted on the outside edges of the TLC plates with conc. H_2SO_4; each band was scraped off and eluted with MeOH. After evaporation of MeOH, the residues of 4-monomethyl sterols and sterones fractions were counted in a liquid scintillation spectrometer. The residues of cholesterol and lanosterol analogs fractions were cocrystallized with the corresponding analogs. Incubations were carried out in duplicate and each value above is the average of two experiments.

had remained unchanged in the incubation. As shown in Table I, the tritium-labeled lanosterol and dihydrolanosterol in the control experiments were converted to tritium-labeled cholesterol to the extents of 24.8 and 23.4%, respectively. The most likely explanation for these results is that natural side chain structure of lanosterol is desirable for further metabolism to cholesterol.

Dihydrolanosterol has been used as a substrate for biosynthetic studies[3,7,9,10] by many workers. As [24-^{3}H]-dihydrolanosterol is converted into [24-^{3}H]-cholesterol at approximately the same rate as [24-^{3}H]-lanosterol, as shown in Table I, [24,25-^{3}H]-27-nor-24,25-dihydrolanosterol was used as the substrate for the investigation of metabolism.

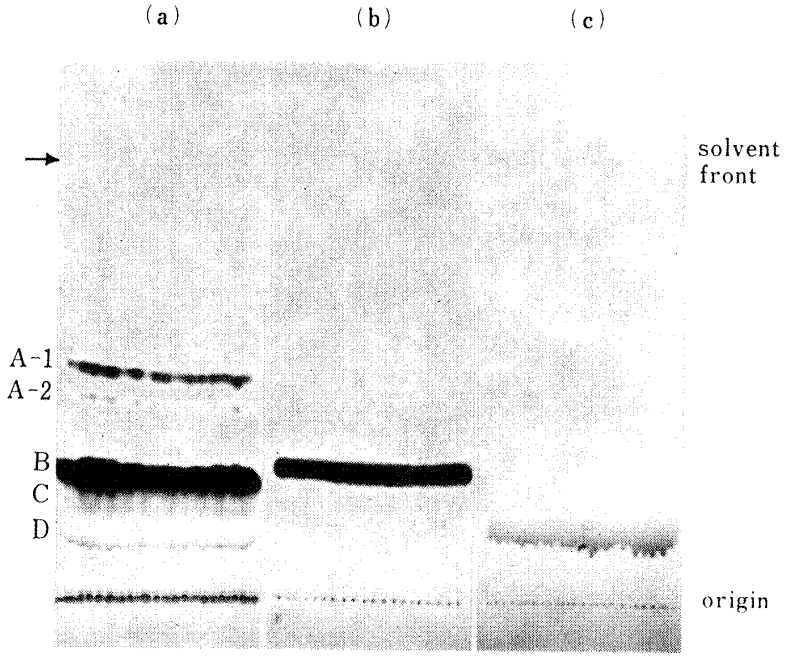


Fig. 1. Radioautographs showing the Distribution of Radioactivity in 27-Nor-cholesterol and its Triterpenoid Precursors after Incubation of a Rat Liver Fraction (S-10) with [24,25-^{3}H]-27-Nor-24,25-dihydrolanosterol

(a) Neutral fraction. (b) After cocrystallization of the eluate from band B with 27-nor-24,25-dihydrolanosterol. (c) After cocrystallization of the eluate from band D with 27-norcholesterol.

The radioautograph of incubation products of [24,25-^{3}H]-27-nor-24,25-dihydrolanosterol is shown in Figure 1a. As judged from their chromatographic properties, bands A-1 and A-2, band B, band C, and band D corresponded to 4-monomethyl sterones and 4,4-demethyl sterones, 4,4-dimethyl sterols, 4-monomethyl sterols, and 4,4-demethyl sterols, respectively.[7,8] The eluate from band B was cocrystallized with 40 mg of 27-nor-24,25-dihydrolanosterol (**1a**) to a constant specific activity (1.05×10^6 dpm/mg), suggesting 42.6% recovery of the starting material (Figure 1b). Similarly, the eluate from band D was cocrystallized with 27-norcholesterol (**3a**, 100 mg); the specific activity of the crystals remained constant during four recrystallizations, that is, 7.24×10^4, 6.29×10^4, 6.68×10^4, and 6.27×10^4 dpm/mg, corresponding to 6.5% transformation of the 27-nor analog into 27-norchloesterol. Furthermore, the value of specific activity corresponds to about 86% of the total activity of band D. The radioautograph is shown in Figure 1c.

The eluate from the sterone fraction (A-2) was cocrystallized with 50 mg of 27-norcholest-5-en-3-one (**9**) to a constant specific activity, suggesting 2.3% conversion to 27-norcholest-5-en-3-one.

In this experiment, the identification of the metabolites of band C could not be performed because authentic 4α-monomethyl-27-norcholesterol derivatives were not available.

In order to obtain further information on the metabolites, parallel incubations of S-10 with unlabeled 27-nor-24,25-dihydrolanosterol (**1a**) were performed on a large scale. After TLC separation of the products, the eluates from each fraction were analyzed by GC–MS. Peaks lacking in blank experiments were found in eluates from the lanosterol fraction, cholesterol fraction and sterone fraction. The new peak in the lanosterol fraction coincided with the starting material. The new peak in the cholesterol fraction coincided with 27-norcholesterol (**3a**); the mass spectra of the peak (the metabolite of the cholesterol fraction) (B) and authentic 27-norcholesterol (A) are compared in Figure 2. These results indicate that the 27-nor analog of dihydrolanosterol is also metabolized by rat liver S-10 fraction to the

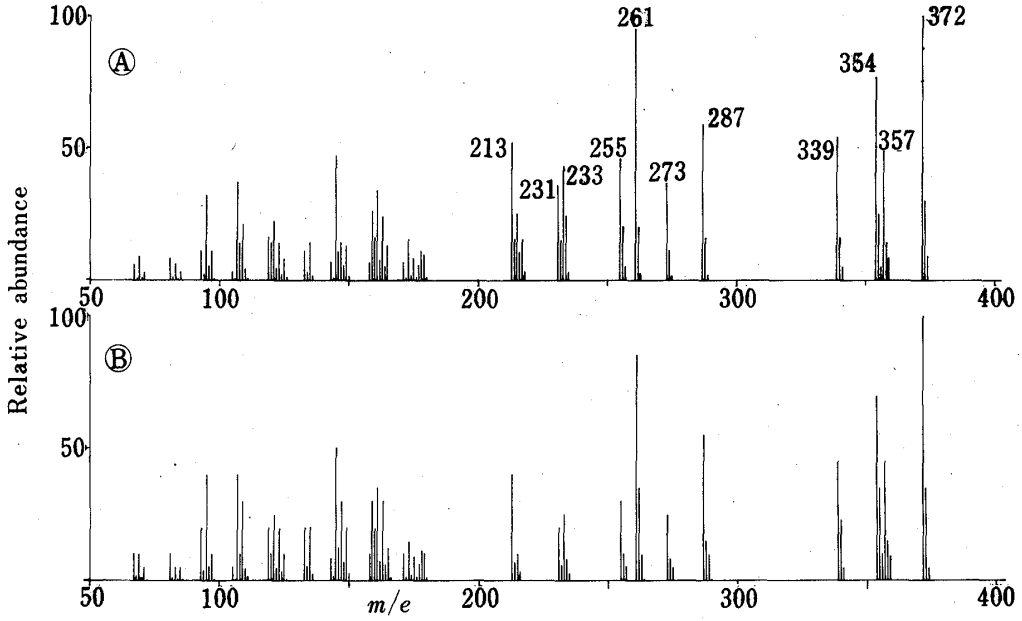


Fig. 2. Mass Spectra of 27-Norcholesterol (Ⓐ) and the Metabolite of the Cholesterol Fraction (Ⓑ)

A coiled glass column (3 mm × 2m) was packed with 1.5% OV-17, and helium was used as the carrier gas. The column temperature and the inlet pressure of carrier gas were 265°C and 1.2 kg/cm², respectively.

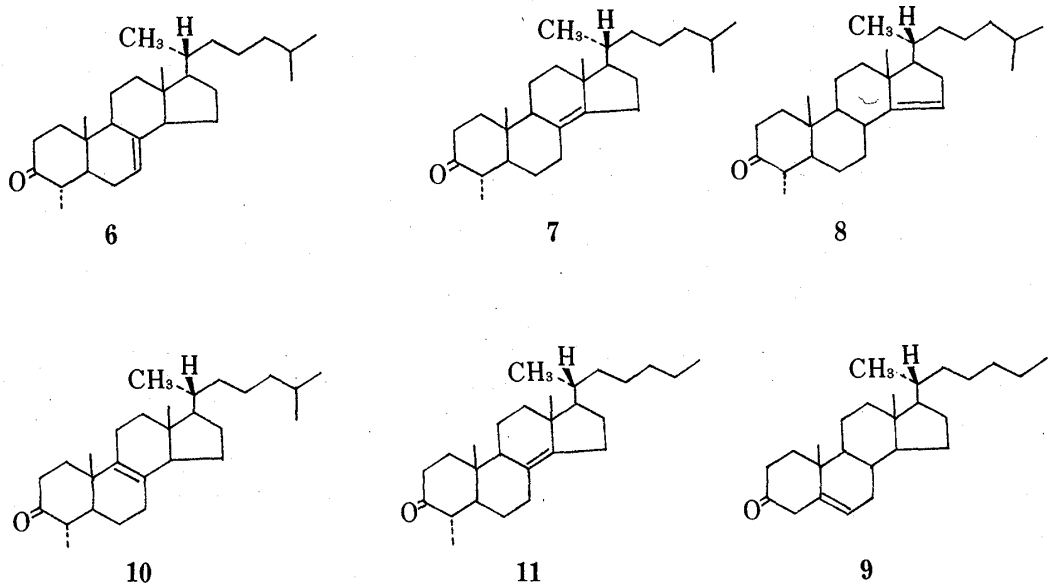


Chart 2

27-nor analog of cholesterol, in spite of its being an inhibitor of cholesterol biosynthesis from lanosterol, in accordance with the results obtained with [24,25-^{3}H]-27-nor-24,25-dihydrolanosterol mentioned above.

Next, the new peak in the sterone fraction corresponding to band A-1 was analyzed by GC–MS, and the metabolite was tentatively assigned as 4α-methyl-27-norcholest-8(14)-en-3-one by comparison of the MS fragmentation patterns of Δ^7, $\Delta^{8(14)}$, and Δ^{14} derivatives of 4α-methylcholestan-3-one (**6**, **7**, **8**) (Chart 2 and Figure 3). The spectrum of the metabolite has five characteristic peaks a, b, c, d and e other than the molecular peak. The peaks a and b can identified[11] as $M^+ - CH_3$ and M^+−side chain, respectively. The peaks c and d may have been formed by loss of the side chain and elision of ring D (loss of C-16 and C-17). The peak e, on the other hand, may have been formed by loss of the side chain and elision of ring D (loss of C-15—C-17). The fragmentation patterns and intensities of the peaks (a—e) are similar to those in the spectrum (B) of 4α-methylcholest-8(14)-en-3-one (**7**). Further, the

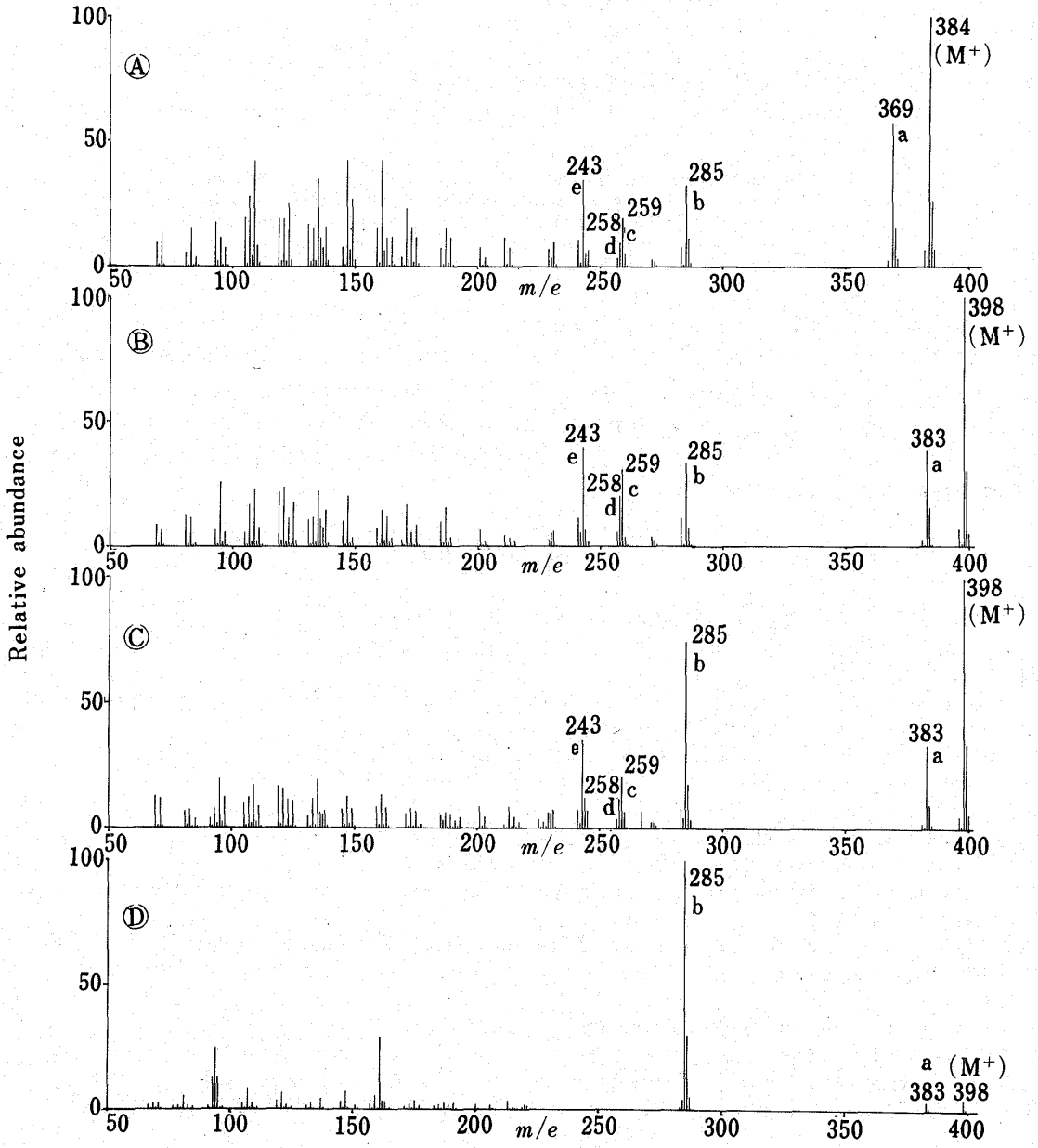


Fig. 3. Mass Spectra of Metabolite (**11**) of the Sterone Fraction and Authentic 4α-Methyl Sterones

Ⓐ: The metabolite (**11**), Ⓑ: 4α-methylcholest-8(14)-en-3-one (**7**), Ⓒ: 4α-methylcholest-7-en-3-one (**6**), Ⓓ: 4α-methylcholest-14-en-3-one (**8**). a: $M^+ - CH_3$, b: M^+−side chain, c: M^+−(side chain+26), *d*: M^+−(side chain+27), e: M^+−(side chain+42).

relative retention time (rt_R) of **7** with respect to cholesterol coincided with that of the metabolite (**11**) to 27-norcholesterol (Table II). Consequently, the structure of the metabolite of the A-1 band (Figure 1a) in the isotope experiments was determined to be 4α-methyl-27-norcholest-8(14)-en-3-one (**11**). To our knowledge, this 4α-methyl-$\Delta^{8(14)}$-sterone analog is the first of its type obtained as a metabolite of lanosterol analogs. In a control experiment using [24-^{3}H]-lanosterol or [24-^{3}H]-dihydrolanosterol as the substrate, no compounds of this type were identified. Further, it is interesting to note that 8(14)-unsaturated sterols have been implicated in the processes of demethylation at C-14 in sterol biosynthesis.[14–16]

Table II. Relative Retention Times (rt_R) of 4α-Methylcholesterones and the Metabolite (**11**) on 1.5% OV-17

4α-Methylcholest-7-en-3-one (**6**)	1.46[a]
4α-Methylcholest-8(14)-en-3-one (**7**)	1.26[a]
4α-Methylcholest-14-en-3-one (**8**)	1.24[a]
4α-Methylcholest-8-en-3-one (**10**)[c]	1.16[a]
The metabolite (**11**) of 27-nor-24,25-dihydrolanosterol (**1a**)	1.26[b]

a) rt_R to cholesterol.
b) rt_R to 27-norcholesterol.
c) This compound was not synthesized, but rt_R to cholesterol was calculated from the data of Katsuki *et al.*[12] and Kato *et al.*[13] Further, comparison of the MS of **11** and 4α-methylcholesta-8,24-dien-3-one,[13] which was characterized as a metabolite of mevalonate, indicated differences in their fragmentation patterns.

On the other hand, no new peak was detected in the A-2 and C bands, probably because of the low yields of 4,4-demethylcholest-5-en-3-one and 4-monomethyl stenol derivatives.

In summary, we found that 27-nor-24,25-dihydrolanosterol is metabolized to 27-norcholesterol, but the pentanor analog of dihydrolanosterol is not metabolized to the corresponding analog of cholesterol. The structure of the side chain thus appears to have a strong influence on the metabolism of lanosterol analogs.

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