Table I. Measured, Derived, and Related Bond Dissociation Energies

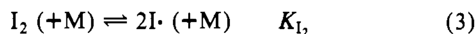
bond	D/ (kJ mol⁻¹)	ref	bond	D/ (kJ mol⁻¹)	ref
$H_3Ge–H$	346	this work	$H_3Si–H$	378	1
$Me_3Ge–H$	340	2	$Me_3Si–H$	378	1
$H_3Ge–GeH_3$	276	this work	$H_3Si–SiH_3$	308	1
$Me_3Ge–GeMe_3$	305	13	$Me_3Si–SiMe_3$	337	14

and other products. Similar behavior has been observed previously in the reaction of SiH_4 with I_2.[8]

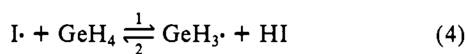
Initial rates were found to fit rate eq 2 for a range of I_2 pressures

$$-d[I_2]/dt = k[I_2]^{1/2}[GeH_4] \quad (2)$$

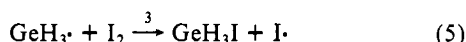
from 0.2 to 3.3 torr and GeH_4 pressures from 2.8 to 58 torr. The data from 20 runs give a value of $k = (4.92 \pm 0.35) \times 10^{-5}$ torr$^{-1/2}$ s^{-1}. Excluding the five runs for which $[GeH_4]_0/[I_2]_0 < 4$ gives a value of $k = (4.78 \pm 0.23) \times 10^{-5}$ torr$^{-1/2}$ s^{-1}. Integrated plots of 2 up to 75% conversion fit the data with the same rate constant, providing $[GeH_4]_0/[I_2]_0 > 100$. Inhibition of the reaction by HI is very slight, and only becomes important at high conversion. These observations are consistent with the mechanism shown in equations 3–5.



$$I_2\ (+M) \rightleftharpoons 2I\cdot\ (+M) \qquad K_{I_2} \quad (3)$$



$$I\cdot + GeH_4 \underset{2}{\overset{1}{\rightleftharpoons}} GeH_3\cdot + HI \quad (4)$$



$$GeH_3\cdot + I_2 \xrightarrow{3} GeH_3I + I\cdot \quad (5)$$

The steady-state expression for this mechanism is eq 6, which

$$-\frac{d[I_2]}{dt} = \frac{k_1 K_{I_2}^{1/2}[I_2]^{1/2}[GeH_4]}{1 + (k_2/k_3)[HI]/[I_2]} \quad (6)$$

reduces to eq 2 in the initial stages of reaction, at which point $k = k_1 K_{I_2}^{1/2}$. From the known value[9] of $K_{I_2}^{1/2}$, $k_1 = (8.21 \pm 0.40) \times 10^4$ dm^3 mol^{-1} s^{-1}. Further measurements of k_1 in the temperature range 402–445 K give the Arrhenius equation (7). The

$$\log (k_1/\text{dm}^3\ \text{mol}^{-1}\ \text{s}^{-1}) = (11.03 \pm 0.13) - (52.3 \pm 1.0\ \text{kJ mol}^{-1})/(RT \ln 10) \quad (7)$$

high A factor for k_1 is in accord with expectations for H atom abstractions by I·.[1,7] This lends further support to the mechanism. The inhibition constant (k_2/k_3) was measured by addition of an excess of HI to the reaction mixture. The value obtained at 445 K is 0.060 ± 0.010, and there is only a very slight temperature dependence. Because of the large scatter and weak temperature dependence, it is difficult to measure the Arrhenius parameters for the ratio k_2/k_3 with reliability, but the data are consistent with $E_2 - E_3 = 4 \pm 4$ kJ mol^{-1}. E_2 cannot be obtained separately, but by analogy with hydrocarbon[7] and silane[1] chemistry E_3 should be zero, and hence $E_2 = 4 \pm 4$ kJ mol^{-1}. Thus $\Delta H_{12}{}^\circ = E_1 - E_2 = 48 \pm 5$ kJ mol^{-1}. With assumption of a negligible effect of temperature, $D(H_3Ge–H) = D(H–I) + (48 \pm 5) = 346 \pm 5$ kJ mol^{-1} (83 ± 1 kcal mol^{-1}). The uncertainty quoted here is based only on an analysis of random error. In the light of the assumptions made to derive E_2, a more realistic estimate of uncertainty might be ±10 kJ mol^{-1}.

The value for $D(H_3Ge–H)$ is within the range previously reported[3–5] but is a good deal lower than the upper limit.[5] It is in disagreement with the electron impact based value[4] but in reasonably good agreement with the results from an IR chemiluminescence study (upper limit originally of 326 kJ mol^{-1},[3] recently revised upward to 338 kJ mol^{-1} [10]). As Table I shows, the value for $D(H_3Ge–H)$ is the same within experimental error as the value previously reported for $D(Me_3Ge–H)$. Thus contrary to the work of McKean et al.[6] there is little or no effect of methyl substitution on the Ge–H bond dissociation energy.[11] This parallels the situation in the analogous silanes.

Finally $D(H_3Ge–H)$ can be used to calculate two other quantities of thermochemical interest. Based on the known values[12] for $\Delta H_f{}^\circ$ of GeH_4 and Ge_2H_6 we derived $\Delta H_f{}^\circ(GeH_3\cdot) = 219$ kJ mol^{-1} and $D(H_3Ge–GeH_3) = 276$ kJ mol^{-1}. The latter is compared with the Ge–Ge dissociation energy of Ge_2Me_6 in the table. Once again there is a striking parallel with the silanes. Methyl groups appear to be acting as *bond strengtheners*, although the effect is only ca. 5 kJ mol^{-1}/methyl group. Since both Ge and Si are relatively electropositive compared with carbon, it is perhaps not too surprising that methyl groups cannot function as inductive electron donors when attached to Ge or Si.

Acknowledgment. We thank the U.K. Science Research Council for support of this work and Professor Don Setser for helpful corrspondence.

Registry No. GeH_4, 7782-65-2; I_2, 7553-56-2; Ge, 7440-56-4; H, 1333-74-0; $H_3Ge\text{-}GeH_3$, 13818-89-8.

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Microbial Degradation of the Phytosterol Side Chain. 1. Enzymatic Conversion of 3-Oxo-24-ethylcholest-4-en-26-oic Acid into 3-Oxochol-4-en-24-oic Acid and Androst-4-ene-3,17-dione

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Cholesterol, sitosterol, and campesterol are abundant naturally occurring sterols, and they have long been recognized as valuable intermediates for steroid hormone production to supplement or supplant the commercial processes from diosgenin.[1] In recent years, several economical microbiological processes[2] have been developed for the conversion of these sterols into useful steroid hormones via the selective cleavage of the hydrocarbon side chain without degrading the steroid nucleus.

In 1968,[3,4] we defined the intermediates and reaction sequence for the microbial transformation of cholesterol into 17-keto steroids. This pathway proceeds via 3-oxochol-4-en-24-oic acid (**1**) and 3-oxobisnorchol-4-en-22-oic acid with the concomitant formation of 2 mol of propionic acid and 1 mol of acetic acid. However, until now the mechanism of degradation of the branched hydrocarbon side chain of sitosterol was not understood. As a first step toward deducing the degradative pathway, we have developed cell-free systems capable of catalyzing the conversion

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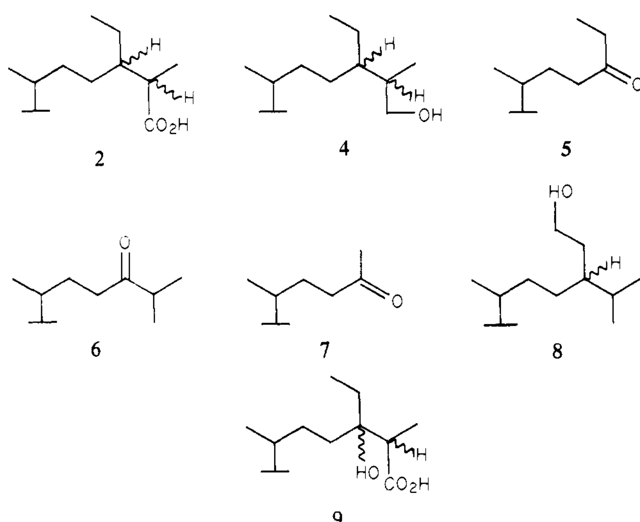
of 3-oxo-24-ethylcholest-4-en-26-oic acid (**2**) into the intermediate



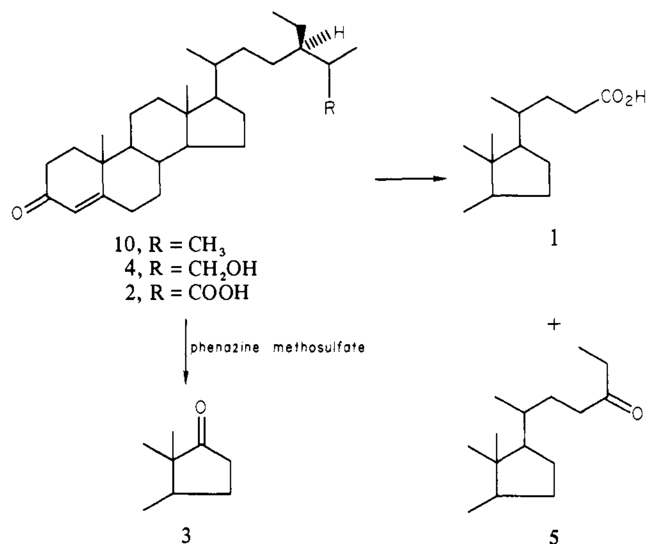
1. Further, in the presence of the electron acceptor phenazine methosulfate, this cell-free system transformed **2** into androst-4-ene-3,17-dione (**3**).

Our initial feeding experiments showed that approximately 2–3% of the radiolabel appeared in **1** after exposure of [4-^{14}C]sitosterol[5] to resting cells of *Mycobacterium sp.*[6] NRRL 3805, indicating that carbon–carbon fission occurred at C-24–C-25 and C-24–C-28. To follow the metabolite fate of the branched chain carbons (C-28 and C-29), we synthesized and incubated [29-^{14}C]sitosterol[7] with the same organism under similar conditions. Unfortunately, most of the radioactivity appeared as $^{14}CO_2$ and only small quantities of radiolabel (0.1–0.5%) were found to be randomly distributed among a number of volatile acids, including acetic acid.

In an attempt to gain further insights into the degradative mechanism, a series of synthetic compounds[8] with modified side chains were prepared and incubated with growing cells of *Mycobacterium sp.* NRRL B-3805. While compounds **2** and **4–6** were all metabolized into **3** at a rate comparable to that of sitosterol, unfortunately no new intermediates were detectable in the medium. Compound **7** was only slowly metabolized into **3**, whereas **8** and **9** were not. These results suggest that a possible degradative sequence may proceed via sitosterol → **4** → **2** → **5** → **1**.

It was reported[9] that a strain of *Mycobacterium smegmatis* when incubated with cholesterol afforded 26-hydroxycholest-4-en-3-one in 0.1–0.2% yield. However, we were unable to obtain workable quantities of the corresponding 26-hydroxy-24-ethylcholest-4-en-3-one (**4**) after exposure of sitosterol to this same organism. On the other hand, we were able to isolate a *Mycobacterium sp.* designated as "4-1" from soil by using pristane as a sole carbon source. When this organism was grown in a medium[10] containing 1% pristane and 1% sitosterol for 48–54 h, 8% of **4** was obtained; mp 106–115 °C;[11] $[\alpha]_D^{25}$ +89.6° (*c* 1.19, $CHCl_3$); NMR ($CDCl_3$) δ 0.70 (s, 3 H, 18-CH_3), 1.19 (s, 3 H, 19-CH_3), 3.44 (dd, J = 7, 11 Hz, 1 H, 26-H_a), 3.60 (dd, J = 6, 11 Hz, 1 H, 26-H_b), 5.73 (br s, 1 H, 4-H). Jones oxidation of **4** furnished the 26-acid **2**: mp 160–161 °C;[12] $[\alpha]_D^{25}$ +71.8° (*c* 1.81, $CHCl_3$); NMR ($CDCl_3$) δ 0.70 (s, 3 H, 18-CH_3), 1.19 (s, 3 H, 19-CH_3), 5.73 (br s, 1 H, 4-H), 10 (br, 1 H, acidic H). The isolation of **4** strongly supports the view that the initial step in the degradative sequence of the sitosterol side chain occurs via hydroxylation at C-26 as is the case for the degradation of cholesterol.[3]

To further define the intermediates and reaction sequence, we turned our attention to the development of active cell-free systems. However, incubation of either sitosterol or sitostenone (**10**) with



cell extracts of *Mycobacterium sp.* NRRL 3805 in the presence of NADPH or NADH afforded only recovered starting material. Because hydroxylation enzymes are often unstable and thus it may be difficult to secure active C-26 hydroxylase preparations, we decided to bypass this obstacle by the use of oxygenated intermediates such as 26-hydroxy-24-ethylcholest-4-en-3-one (**4**) or 3-oxo-24-ethylcholest-4-en-26-oic acid (**2**) as substrates for enzymatic incubations.

When **2** (200 μg) was incubated with the 100000*g* supernatant fraction[13] (2.5 mL, 30 mg of protein) of the cell extract of *Mycobacterium sp.* NRRL 3805 in the presence of 5 μmol of ATP, 10 μmol of $MgCl_2$ and 2.5 μmol of coenzyme A in 0.05 M phosphate buffer, pH 7.8 for 60 min at 25 °C, a major acidic compound (80% yield) having chromatographic (TLC[14] and HPLC[15]) and spectroscopic properties identical with those of **1** was formed. In addition a minor product with properties corresponding to 27-norcholest-4-ene-3,24-dione (**5**) was obtained in 3% yield. It is noteworthy that 9α-hydroxy-27-norcholest-4-

(5) [4-^{14}C]Sitosterol (58 mCi/mmol) was purchased from Amersham.

(6) This bacterium was selected for our studies because it lacks the Δ^1-dehydrogenase and possesses low levels of 9α-hydroxylase. Hence, it degrades the steroid nucleus at a negligible rate.

(7) This radioactive sterol (0.33 mCi/mmol) was synthesized by the reaction of 24-formylcholest-5-en-3β-ol THP ether with [^{14}C]CH_3PPh_3I ([^{14}C]CH_3I, 58 mCi/mmol, Amersham): Fujimoto, Y.; Sih, C. J., unpublished work. This compound is epimeric at C-24.

(8) It should be noted that some of the synthetic compounds consist of a mixture of stereoisomers: compounds **2**, **4**, and **9** are a mixture of four isomers, each diastereomeric at C-24 and C-25. Compound **8** is an epimeric mixture at C-24. All compounds possess the 3-oxo-4-ene structures except **8** (Δ^5, 3-OH).

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(11) The sample of **4**, obtained from fermentation, appeared to be a single stereoisomer at C-24 and C-25 from the relatively simple NMR pattern. Also, cholesterol gave (26*S*)-26-hydroxycholest-4-en-3-one.[9] However, the mass spectrum of **4** revealed that **4** (M^+, 428) is contaminated with a small amount of 26-hydroxy-24-methylcholest-4-en-3-one (M^+, 414), derived from campesterol.

(12) Repeated crystallization of **2** from ethyl acetate afforded pure **2**.

(13) *Mycobacterium sp.* NRRL B-3805 cells were suspended in 10 volumes of 0.05 M phosphate buffer, pH 7.8. This suspension was sonicated with a Bronson sonifier (20 kHz) for 5 min in an ice bath. The crude extract was freed of whole cells and debris by centrifugation at 40000*g* for 10 min. The supernatant was further centrifuged at 100000*g* for 30 min. The 100000*g* supernatant fraction was used for our experiments.

(14) Brinkmann (EM) plates coated with 0.25 mm thickness of silica gel containing PF254. The developing solvent system: Skelly B–ethyl acetate–acetic acid (80:40:1). The R_f values are as follows: **1**, 0.30; **2**, 0.37; **3**, 0.19; **5**, 0.48.

(15) HPLC separation was effected on a Waters radial compression module (RCM-100) using a radial-Pak 5-μm silica gel cartridge (0.8 × 10 cm) with hexane–$CHCl_3$ (2:1) as the mobile phase at a flow rate of 2 mL/min. Retention times: **3** = 38 min; **5** = 17.2 min; **1** methyl ester = 16.5 min.

ene-3,24-dione and 9α-hydroxy-26,27-bisnorcholest-4-ene-3,24-dione had been isolated from the fermentation broth after incubation of sitosterol and campesterol with a blocked mutant of *Mycobacterium fortuitum*.[16]

When the above cell-free system was supplemented with the electron acceptor phenazine methosulfate (2.5 μmol), all of **2** was quantitatively transformed under similar conditions into a 17-keto steroid having chromatographic (TLC[14] and HPLC[15]) and spectroscopic (UV, MS) properties corresponding to **3**. Because **5** was rapidly metabolized into **3** by intact cells of *Mycobacterium sp.* NRRL B-3805, one might surmise that **5** might be an intermediate in the reaction pathway. However, when either **5** or **7** was exposed to the above cell-free system containing phenazine methosulfate, they were recovered unchanged. This result conclusively established that **5** and **7** are not intermediates of the main degradative pathway. Although the exact mechanism of formation of **5** from sitosterol is yet to be resolved, one can envisage that **5** may originate nonenzymically from an unstable β-keto acid intermediate via decarboxylation. Alternatively, it may be derived via a scavenger pathway involving reverse aldolytic cleavage of the β-hydroxy coenzyme A derivative (see Scheme I of ref 17).

Our investigations clearly demonstrate that the mode of microbial degradation of the sitosterol side chain proceeds via hydroxylation at C-26, followed by oxidation to **2**, which is transformed into **3** via the intermediate **1**. The availability of an active cell-free system for the conversion of **2** into **1** allows one to define the key metabolic reactions taking place prior to carbon–carbon fission. This constitutes the subject of the accompanying communication.[17]

Acknowledgment. We thank Dr. E. Caspi for a transfer of *Mycobacterium smegmatis*. This investigation was supported in part by Grant GM 26838 of the National Institutes of Health.

Registry No. 1, 1452-29-5; **2**, isomer 1, 82537-06-2; **2**, isomer 2, 82570-86-3; **2**, isomer 3, 82537-07-3; **2**, isomer 4, 82570-87-4; **3**, 63-05-8; **4**, isomer 1, 82537-13-1; **4**, isomer 2, 82570-91-0; **4**, isomer 3, 82537-15-3; **4**, isomer 4, 82570-92-1; **5**, 82537-14-2; **6**, 57701-41-4; **7**, 82537-05-1; sitosterol, 83-46-5; pristane, 1921-70-6.

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Microbial Degradation of the Phytosterol Side Chain. 2. Incorporation of $NaH^{14}CO_3$ onto the C-28 Position

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In the previous communication,[1] we established the intermediacy of 3-oxochol-4-en-24-oic acid (**1**) during the microbial conversion of sitosterol into 17-keto steroids. We herein report the metabolic fate of the branched carbons C-28 and C-29 of sitosterol and C-28 of campesterol and demonstrate that HCO_3^- is incorporated onto the C-28 position of these phytosterols prior to carbon–carbon bond fission.

Exposure of [28-^{14}C]-3-oxo-24-ethylcholest-4-en-26-oic acid[2] (**2**) to the 100000*g* supernatant fraction[1] (5 mL, 60 mg of protein) of *Mycobacterium sp.* NRRL B-3805 in the presence of ATP (10 μmol), coenzyme A (5 μmol), and $MgCl_2$ (20 μmol) in 0.05 M phosphate buffer, pH 7.8 for 90 min resulted in the formation of a radioactive volatile acid (25% incorporation). Its behavior on a Celite-535 partition column[3] was identical with that of propionic acid. The product was identified by admixture with nonisotopic propionic acid and converted to the *p*-bromophenacyl derivative,[4] mp 62.5–63 °C; its specific activity remained essentially constant after three recrystallizations. Further, the HPLC[5] retention time of the isotopically labeled and authentic *p*-bromophenacyl propionate was found to be identical (9.4 min). Schmidt degradation[6] of the propionic acid revealed that the 2-carbon of the molecule contained all of the radioactivity. This experiment suggested that bicarbonate ion may have been incorporated onto either the C-23 or the C-28 position of **2**. To distinguish these two possibilities, we incubated **2** with the same cell-free system in the presence of $NaH^{14}CO_3$.[7] In this instance, approximately 5% of the radioactivity was found in propionic acid. All of the radioactivity resided in the 1-carbon as revealed by Schmidt degradation.[6] Also, the resulting steroidal fragment **1** was devoid of radioactivity. These results clearly indicate that HCO_3^- was incorporated onto the C-28 position of **2**.

Since the soya sterols contain a mixture of sitosterol and campesterol in a ratio of 3:2,[8] we should also like to establish the mechanism via which the campesterol side chain is degraded by microorganisms. Because of the relative scarcity of pure campesterol, we were unable to prepare the corresponding 26-hydroxy-24-methylcholest-4-en-3-one (**3**) via hydroxylation of campesterol by *Mycobacterium sp.* "4-1". Hence 3-oxo-24-methylcholest-4-en-26-oic acid (**4**) (Chart I) was synthesized as a mixture of four diastereomers via the following sequence of reactions. Treatment of **5**[9] with methylmagnesium iodide in ether (4 equiv, 2 h, 25 °C) afforded the alcohol **6** in 90% yield. The latter was transformed into the bromide **7** (CBr_4, Ph_3P, pyridine, 0 °C, 3 h) in 79% yield; NMR ($CDCl_3$) δ 1.70 (d, 3 H), 4.0 (m, 2 H), 4.70 (m, 1 H), 5.35 (m, 1 H). When **7** was heated in THF at 70 °C with an excess of the anion of diethyl methylmalonate (16 equiv), slow alkylation occurred (3–4 days) to yield the diester **8** (81%); NMR δ 0.88 (d, 6 H), 1.29 (s, 3 H), 4.15 (q, 2 H). When **8** was heated with 4 equiv of NaCN in Me_2SO for 10 h at 160 °C, clean decarboethoxylation occurred to give **9** (65%); NMR δ 0.92 (d, 6 H), 1.02 (s, 3 H), 4.15 (q, 2 H). After cleavage of the THP protecting group, the resulting hydroxy ester **10** was saponified ($EtOH/KOH/H_2O$, 70 °C, 11 h) to afford the acid **11**. Oppenauer oxidation of **11** afforded **4** (64%); NMR δ 0.67 (s, 3 H), 1.16 (s, 3 H), 5.72 (s, 1 H).

When **4** was incubated (Chart II) with the cell-free system of *Mycobacterium sp.* NRRL B-3805 under similar conditions, **1** was isolated in approximately 50% yield, accompanied by a trace quantity of 26,27-bisnorcholest-4-ene-3,24-dione (**12**). If phenazine methosulfate was included in the cell-free system, **4** was transformed into androst-4-ene-3,17-dione (**13**) as was in the case of **2**.

If **4** is degraded by a mechanism similar to that of **2**, radioactive HCO_3^- should likewise be incorporated onto the C-28 position of **4** and the radiolabel should reside in acetic acid. In accord with this prediction, when $NaH^{14}CO_3$ was incubated with cell extracts of *Mycobacterium sp.* NRRL B-3805 and **4**, a volatile acid with chromatographic properies on a Celite-535 partition column[3] coinciding with that of acetic acid was obtained. The product was identified by admixture with nonisotopic acetic acid

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(2) The radioactive acid, **2** (0.22 mCi/mmol), was synthesized from 3β-hydroxycholenic acid (Fujimoto, Y.; Sih, C. J., unpublished work). The ^{14}C was introduced by reaction of 3β-tetrahydropyranyloxychol-4-en-24-al with [1-^{14}C]ethylmagnesium iodide ([1-^{14}C]ethyl iodide was purchased from Amersham, 57.4 mCi/mmol). It should be noted that this synthetic **2** consisted of a mixture of four isomers, diastereomeric at C-24 and C-25.
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(7) The $NaH^{14}CO_3$ was purchased from New England Nuclear (52.5 mCi/mmol).
(8) Commercial sitosterol (Aldrich) was analyzed by GLC (OV-1 on 3% Chromosorb WHP, 3 h, 275 °C). Retention times were as follows: sitosterol, 7.9 min; campesterol, 6.8 min.
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