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.

(16) Knight, J. C.; Wovcha, M. G. *Steroids* **1980**, *36*, 723.
(17) Fujimoto, Y.; Chen, C.-S.; Gopalan, A.; Sih, C. J. *J. Am. Chem. Soc.*, following communication in this issue.

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

(1) Fujimoto, Y.; Chen, C.-S.; Szeleczky, Z.; DiTullio, D.; Sih, C. J. *J. Am. Chem. Soc.*, preceding paper in this issue.
(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.
(3) Swim, H. F.; Utter, M. F. *Methods Enzymol.* **1957**, *4*, 584.
(4) Vogel, A. I. "A Textbook of Practical Organic Chemistry", 3rd ed.; Wiley: New York, 1956; p 362.
(5) 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.
(6) Abraham, J. K. S.; Charkoff, I. L. *Anal. Chem.* **1955**, *27*, 155.
(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.
(9) Koizumi, N.; Morisaki, M.; Ikekawa, N. *Tetrahedron Lett.* **1978**, 2899.

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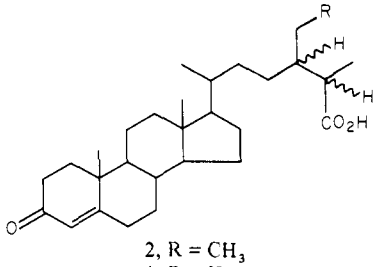
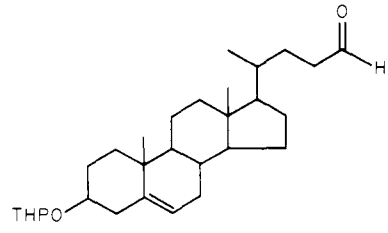
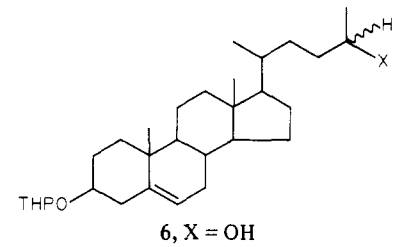
Chart I



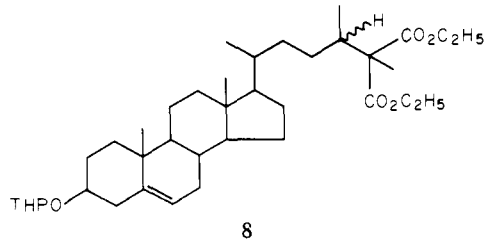
2, R = CH_3
4, R = H



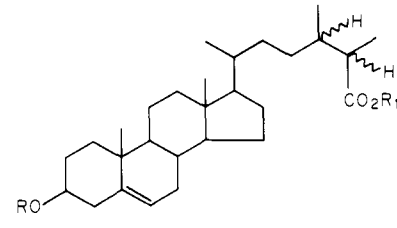
5



6, X = OH
7, X = Br

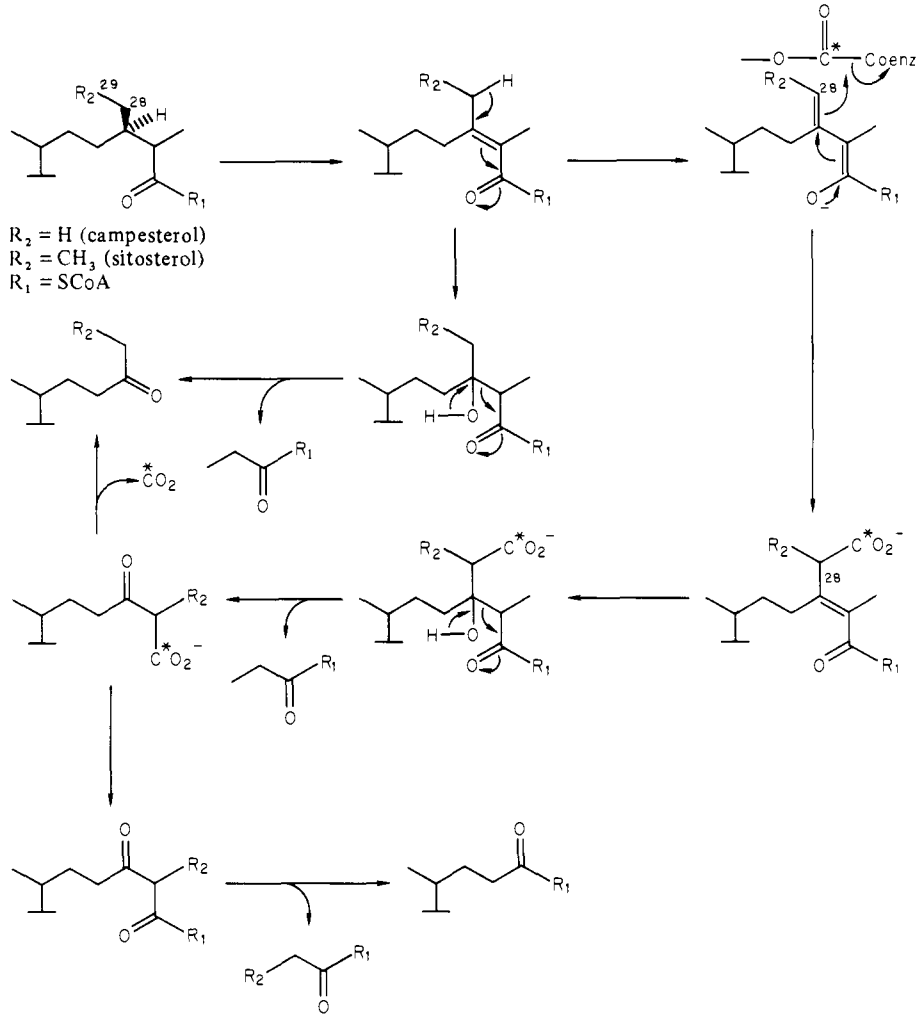


8



9, R = THP; $R_1 = C_2H_5$
10, R = H; $R_1 = C_2H_5$
11, R = R_1 = H

Scheme I

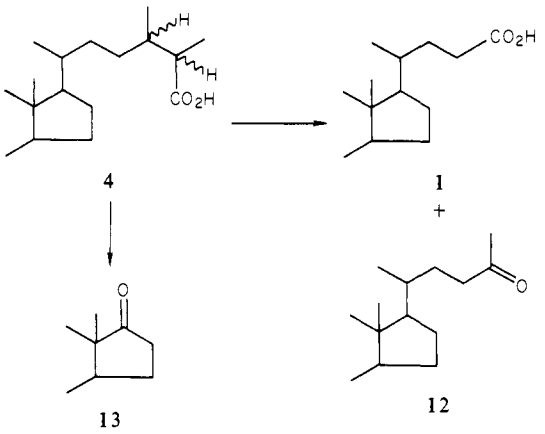


R_2 = H (campesterol)
R_2 = CH_3 (sitosterol)
R_1 = SCoA

and converted to the *p*-bromophenacyl derivative, mp 83–84 °C; its specific activity remained constant after three recrystallizations. Schmidt degradation[6] of the radioactive acetic acid showed that the 1-carbon of the molecule contained most of the label.

These data support the view that the cleavage of the phytosterol side chains by *Mycobacterium sp.* involves the incorporation of 1 mol of HCO_3^- onto the C-28 position of the sterols, followed by carbon–carbon fission at C-24–C-25 and C-24–C-28, resulting in the formation of **1** and 2 mol of propionic acid for sitosterol, whereas campesterol is converted into **1** with the concomitant formation of 1 mol each of acetic and propionic acid. A plausible degradative pathway is shown in Scheme I.

Chart II



This degradative mechanism of the branched side chain carbons (C-28 and C-29) of phytosterol differs from that of the insect system, which cleaves the C-24–C-28 bond via dehydrogenation, epoxidation, and fragmentation to yield desmosterol and acetaldehyde.[10]

Acknowledgment. This research was supported in part by a grant from the National Institutes of Health (GM-26838).

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; **4**, isomer 1, 82537-00-6; **4**, isomer 2, 82570-88-5; **4**, isomer 3, 82570-89-6; **4**, isomer 4, 82537-08-4; **5**, 66414-44-6; **6**, 66414-45-7; **7**, 82537-01-7; **8**, 82537-02-8; **9**, 82537-03-9; **10**, 82537-04-0; **11**, 74730-06-6; **12**, 82537-05-1; **13**, 63-05-8; sitosterol, 83-46-5; campesterol, 474-62-4; propionic acid, 79-09-4; bicarbonate, 71-52-3; acetic acid, 64-19-7.

(10) Ikekawa, N.; Fujimoto, Y.; Takasu, A.; Morisaki, M. *J. Chem. Soc., Chem. Commun.* **1980**, 709 and references cited therein.

Symmetrical Intermediates in C_9H_{12} Biradical Rearrangements. Possible Intervention of an Organic Tetraradical

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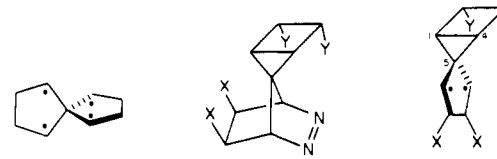
The present work addresses the possibility of generating and characterizing 1,4,6,9-spiro[4.4]nonatetrayl (**1**), an organic tet-



raradical. The singly occupied p orbitals of **1** interact extensively via spiroconjugation,[2] as shown by the orbital mixing diagram in

(1) NSF Predoctoral Fellow, 1980–1983.

(2) Simmons, H. E.; Fukunaga, T. *J. Am. Chem. Soc.* **1967**, *89*, 5208–15. Hoffmann, R.; Imamura, A.; Zeiss, G. D. *Ibid.* **1967**, *89*, 5215–20. Duerr, H.; Gleiter, R. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 559–69.

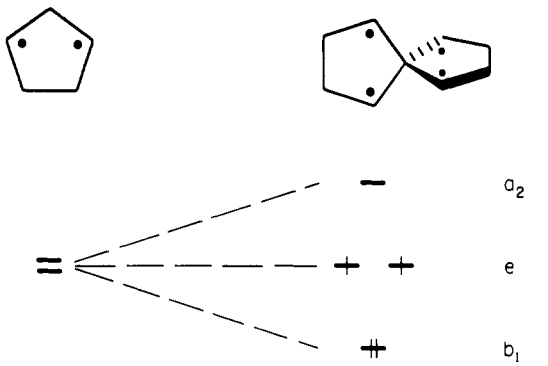


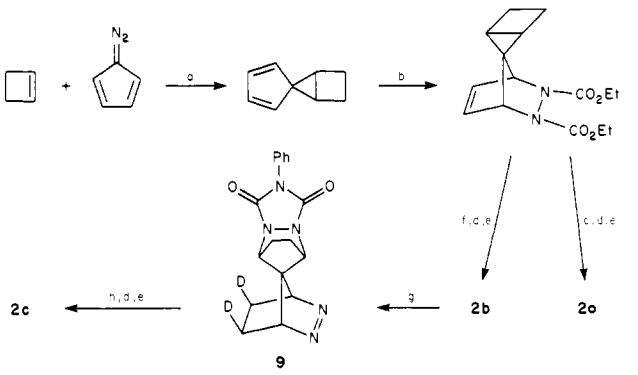
Figure 1. MO interaction diagram showing the effects of spiroconjugation in **1**.

Table I. Product Yields from 2

conditions	4	5	other
140 °C, 4 h	70.6[a]	29.4[a]	
$h\nu$, direct	86.7	9.6	3.6
$h\nu$, Ph_2CO sensitized	2.3	84.6[b]	13.0[b]

[a] Control experiments indicate that 4 → 5 under these conditions. Values are for 90% conversion of 2. [b] These products decompose slowly under the reaction conditions. Values are for 90% conversion of 2.

Scheme I



[a] $h\nu$, $\lambda > 425$ nm. [b] $EtO_2CN{=}NCO_2Et$. [c] $N_2H_4{\cdot}H_2O$, O_2. [d] KOH, *i*-PrOH. [e] O_2. [f] $N_2D_4{\cdot}D_2O$, O_2. [g] PTAD. [h] $h\nu$, $\lambda > 300$ nm, solid state.

Figure 1. Schweig's empirical formula for estimation of the spiroconjugative split in spiro[4.4]nonane derivatives predicts a 1.96-eV gap between the b_1 and a_2 molecular orbitals of **1** (D_{2d} symmetry).[3] Ab initio calculations[4] are fully consistent with this result. Thus, in a structural sense **1** is a tetraradical (two broken bonds),[5] but the electronic structure is that of a biradical (two electrons in a degenerate pair of nonbonding MO's).[6] The substantial energy lowering of the b_1 orbital could significantly stabilize **1** relative to a system containing four noninteracting radical centers.

These and other qualitative considerations led us to speculate that azoalkane **2** could give rise to novel chemistry indicative of **1**. Homolysis of the C1–C4 bond in the biradical **3** obtained upon N_2 loss from **2** relieves ca. 50 kcal/mol of strain energy and allows the full spiroconjugative stabilization of **1** to develop. Thus, the novel biradical → tetraradical sequence **3** → **1** seems feasible.

The synthesis of diazene **2** is outlined in Scheme I.[7] As shown

(3) Schweig, A.; Weidner, U.; Hill, R. K.; Cullison, D. A. *J. Am. Chem. Soc.* **1973**, *95*, 5426–7. This analysis assumes coefficients of $1/2^{1/2}$ for the radical p orbitals in the cyclopentanediyl fragments.

(4) McElwee-White, L.; Goddard, W. A., III; Dougherty, D. A., to be submitted for publication.

(5) Berson, J. A. *Acc. Chem. Res.* **1978**, *11*, 446–53.

(6) Salem, L.; Rowland, C. *Angew. Chem., Int. Ed. Engl.* **1972**, *11*, 92–111.

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