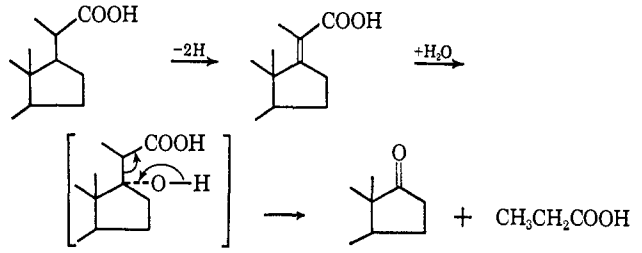
Table I. Propionate-^{14}C from 3-Hydroxybisnorchol-17(20)-en-22-oic Acid-22-^{14}C

S-Benzylisothiuronium propionate, mp 154–155° No. of recrystallization	Spec act., counts/min mmole
First	17,750
Second	19,050
Third	18,620
Distribution of ^{14}C in propionate	**Radioactivity, counts/min**
Sodium propionate	6500
Carbon 1	6260
Carbons 2 and 3 (as ethylamine)	0

oic acid-22-^{14}C was synthesized *via* a similar sequence of reactions as outlined, except 3β-acetoxy-5α-pregnan-20-one was used as the starting material. When it was exposed to washed cells of *Nocardia sp.* (ATCC 19170), a radioactive volatile acid, whose chromatographic behavior on a Celite column[13] was identical with that of propionic acid, was obtained. It was isolated as its S-benzylisothiuronium salt, mp 154–155°; its specific activity remained constant after three recrystallizations. Degradation of the propionic acid molecule[14] revealed that all the radioactivity resided in the carboxyl carbon of propionic acid (Table I).

The data reported herein clearly show that the degradation of the hydrocarbon side chain of cholesterol proceeds *via* C_{22} acid intermediates, which confirms the finding of Whitmarsh. Since 3-oxobisnorchol-4-en-22-oic acid and 3-oxobisnorchol-17(20)-en-22-oic acid could be converted into androst-4-ene-3,17-dione by these microorganisms under *anaerobic* conditions, one may envisage the degradation of the three-carbon side chain involving dehydrogenation, hydration, and aldolytic fission.[15]



(13) S. P. Colowick and N. O. Kaplan, *Methods Enzymol.*, **4**, 584 (1957).
(14) E. F. Phares, *Arch. Biochem. Biophys.*, **33**, 173 (1951).
(15) This investigation was supported in part by research grants from the National Institutes of Health (AM-4874 and AM-6110) and the National Science Foundation (GB-1903).

Charles J. Sih, K. C. Wang, H. H. Tai
School of Pharmacy, University of Wisconsin
Madison, Wisconsin 53706
Received February 3, 1967

The Mechanism of Microbial Conversion of Cholesterol into 17-Keto Steroids

Sir:

In the previous communication, we established the participation of C_{22} acid intermediates in the microbiological transformation of cholesterol into 17-keto steroids. We herein report the reactions leading to the formation of C_{22} acid intermediates from cholesterol (C_{27}), thus completing the degradative sequence of the hydrocarbon side chain.

Exposure of cholesterol-26,27-^{14}C to cells of *Nocardia restrictus* (ATCC 14887) resulted in the formation of a radioactive volatile acid. Its chromatographic behavior on a Celite column[2] was identical with that of propionic acid. The product was identified by admixture with nonisotopic propionic acid and crystallized as its S-benzylisothiuronium salt, mp 153–155°; the specific activity remained essentially constant after three recrystallizations. Degradation of the propionic acid[3] revealed that carbons 1 and 3 of the molecule contained all of the radioactivity in a ratio of 1:1 (Table I). This further substantiates that the radioactive propionic acid is derived from the terminal isopropyl portion of the hydrocarbon side chain.

Table I. Propionate-^{14}C from Cholesterol-26,27-^{14}C

S-Benzylisothiuronium propionate, mp 153–155° No. of recrystallization	Specific activity, counts/min mmole
First	33,450
Second	29,750
Third	29,800
Distribution of ^{14}C in propionate	**Radioactivity, counts/min**
Sodium propionate	2260
Carbon 1	1078
Carbon 2	24
Carbon 3	710

To demonstrate the formation of the steroidal counterpart (C-24 acid), radioactive cholesterol-4-^{14}C was incubated with cells of *N. restrictus*, in the presence of 10^{-3} *M* *o*-phenanthroline[4] and 3-oxochol-4-en-24-oic acid as carrier. After incubation for 4 hr, the radioactive products were isolated by paper chromatography.[5] This system gives a complete separation of cholesterol (R_f 0.9) from 3-oxochol-4-en-22-oic acid (R_f 0.44). The radioactive acid was combined with nonisotopic 3-oxochol-4-en-24-oic acid, and the specific activity after three crystallizations remained stable (90 counts/min mg). These results are all consistent with the formation of a C_{24} acid intermediate *via* fission of the C-24–C-25 bond, in a manner analogous to the conversion of cholesterol into bile acids in mammals.[6] To follow the metabolic fate of the C_{24} acid, lithocholic acid-24-^{14}C was exposed to cells of *N. restrictus*. In this case, a radioactive volatile acid having chromatographic behavior[2] similar to acetic acid was obtained. By admixture with nonisotopic acetic acid it was crystallized as its S-benzylisothiuronium salt, mp 138–141.5°, whose specific activity remained unchanged after several

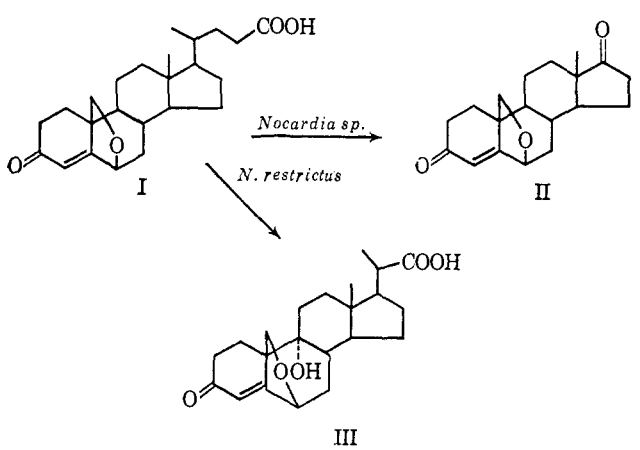
(1) C. J. Sih, K. C. Wang, and H. H. Tai, *J. Am. Chem. Soc.*, **89**, 1956 (1967)
(2) S. P. Colowick and N. O. Kaplan, *Methods Enzymol.*, **4**, 584 (1957).
(3) E. F. Phares, *Arch. Biochem. Biophys.*, **33**, 173 (1951).
(4) *o*-Phenanthroline is an inhibitor of 9α-hydroxylase (unpublished data).
(5) A. Zaffaroni, R. B. Burton, and E. H. Keutman, *Science*, **111**, 6 (1950).
(6) H. M. Suld, E. Staple, and S. Gurin, *J. Biol. Chem.*, **237**, 338 (1962).

Table II. Acetate-^{14}C from Lithocholic Acid-24-^{14}C

S-Benzylisothiuronium acetate, mp 138–141.5°	
No. of recrystallization	Specific activity, counts/min mmole
First	14,460
Second	14,760
Third	15,360
Fourth	15,960
Distribution of ^{14}C in acetate	
	Radioactivity, counts/min
Sodium acetate	3410
Carbon 1	3280
Carbon 2	3

recrystallizations. Schmidt degradation[3] of the molecule revealed that the radioactivity resided exclusively in the carboxyl carbon (Table II).

When 3-oxo-6,19-oxidochol-4-en-24-oic acid[7] (I; 200 mg) was incubated with *Nocardia sp.*[8] (ATCC 19170), 6,19-oxidoandrost-4-ene-3,17-dione (II; 30 mg), mp 182–185°, was obtained. On the other hand, when I (1.5 g) was exposed to *N. restrictus*, 353 mg of a product (III) was isolated: mp 231–234°; $[\alpha]^{26}D$ −66° (dioxane); λ_{max}^{alc} 241 mμ (ε 11,000); λ_{max}^{nujol} 3.00, 5.82, 5.90, and 6.06 μ. Its nmr[9] spectrum showed bands at τ 4.11 (1 H, singlet, vinylic H at C-4), 5.39 (1 H, doublet, J = 5 cps, H at C-6), 5.93 (doublet) and 6.60 (doublet) (2 H, J = 10 cps, CH_2O at C-19), 8.90 (3 H, doublet, J = 8 cps, 21-CH_3), and 9.26 (3 H, singlet, 18-CH_3). Molecular weight analysis (mass spectrum) gave 374 and carbon and hydrogen analysis was in good agreement with $C_{22}H_{30}O_5$. Since III remained unchanged after treatment with pyridine and acetic anhydride or chromic trioxide in acetic acid, the newly introduced hydroxyl group must occupy a tertiary position. On the basis of the deshielding effect, caused by this newly introduced hydroxyl group on the vinylic proton band at C-4 (−0.16 ppm),[10] and the fact that *N. restrictus* is a known 9α-hydroxylator,[11] III was tentatively assigned the structure 9α-hydroxy-6,19-oxidobisnorchol-4-en-3-on-22-oic acid.



(7) This compound was synthesized by the hypoiodite reaction according to the sequence reported by J. Kalvoda, *et al.*, *Helv. Chim. Acta*, 46, 1361 (1963).

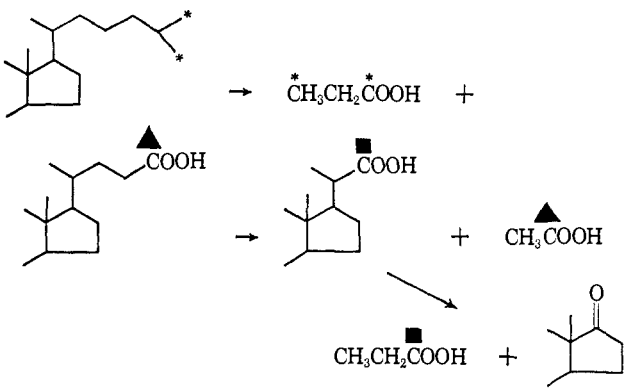
(8) This organism was formerly named CSD-10.

(9) Nuclear magnetic resonance spectra were determined on a Varian Associates recording spectrometer (A-60A) at 60 Mc in deuterated dimethyl sulfoxide. Chemical shifts are reported in τ values (parts per million) [G.V.D. Tiers, *J. Phys. Chem.*, 62, 1151 (1958)].

(10) K. Tori and E. Kondo, *Steroids*, 4, 713 (1964).

(11) C. J. Sih, *Biochim. Biophys. Acta*, 62, 541 (1962).

These data support the view that the degradation of the cholesterol side chain by microorganisms[12] involves carbon–carbon bond fission at C-24–C-25, C-22–C-23, and C-17–C-20, resulting in the formation of 2 moles of propionic acid and 1 mole of acetic acid.



This mode of formation of 17-keto steroids from cholesterol differs from that of the mammalian system[13] which involves the cleavage of the C-20–C-22 bond, yielding isocaproic acid and pregnenolone; subsequent breakage of the C-17–C-20 bond gives 17-keto steroids.[13,14]

(12) Similar results were obtained with other microorganisms of the genera *Mycobacterium*, *Corynebacterium*, and *Arthrobacter*, indicating this to be a general degradative pathway.

(13) K. Shimizu, M. Gut, and R. I. Dorfman, *J. Biol. Chem.*, 237, 699 (1962).

(14) This investigation was supported in part by research grants from the National Institutes of Health (AM-4874 and AM-6110) and the National Science Foundation (GB-1903).

Charles J. Sih, H. H. Tai, Y. Y. Tsong
School of Pharmacy, University of Wisconsin
Madison, Wisconsin 53706
Received February 3, 1967

Automerization of Naphthalene in the Presence of Aluminum Chloride

Sir:

We wish to report preliminary results concerning a new reaction of naphthalene (I): isotopic scrambling of the ^{14}C label from position α into positions β and γ (*i.e.*, the bridge carbon atoms 9 and 10) under the influence of aluminum chloride.

Naphtalene-1-^{14}C (1 mole) was heated in benzene (7 moles) for 2 hr at 60° with commercial anhydrous aluminum chloride (0.95 mole), exposed to the atmosphere during grinding in the mortar. After hydrolysis and removal of the solvent, the recovered and purified naphthalene (about half the initial amount was obtained by distillation) was nitrated by nitric acid in acetic acid, then reduced with zinc powder in ethanol–acetic acid to 1-naphthylamine (II). The attempt to perform a systematic degradation by conversion of II into benzo[*h*]quinoline *via* a Skraup reaction and by subsequent oxidation and decarboxylation failed, because benzo[*h*]quinoline does not afford quinolinic acid on oxidation with potassium permanganate. Therefore 1-naphthylamine was oxidized by potassium permanganate to phthalic acid (III) which was converted into carbon dioxide (IV) and anthranilic acid (V) by a Schmidt degradation.