When VIII was exposed to cell-free extracts of *N. restrictus* it was very readily converted into V, m.p. 98–100°, identical (mixture melting point and infrared spectrum) with an authentic specimen[2] of 3aα-H-4α-[3′-propionic acid]-7aβ-methylhexahydro-1,5-indandione. Since, under similar conditions, IV and VI were only slowly converted into V by these same extracts, VIII appears to be involved as an intermediate in the main pathway of this degradation. Similar conversions have been obtained with other microorganisms[10] indicating that this is a general pathway of steroid degradation.[15]

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Charles J. Sih, S. S. Lee
Y. Y. Tsong, K. C. Wang
School of Pharmacy, University of Wisconsin
Madison 6, Wisconsin
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On the Mechanism of Ring A Cleavage in the Degradation of 9,10-Seco Steroids by Microorganisms

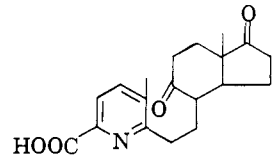
Sir:

In our previous communication[1] we have shown that 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (I) was readily transformed into 3aα-H-4α-[3′-propionic acid]-7aβ-methylhexahydro-1,5-indandione (II) by cell-free extracts of *Nocardia restrictus* (ATCC 14887). We now wish to present evidence for the structure of other intermediates and to define the reaction sequence involved in the cleavage of the steroid A ring.

In a typical experiment, 300 mg. of I was incubated with cell-free extracts of *N. restrictus* in the presence of Fe^{2+}. After acidification, the reaction mixture was extracted overnight with ether. The residue obtained from the ethereal extract was chromatographed over a silicic acid column to yield 62 mg. of II and 40 mg. of an oil (III) whose properties were consistent with 2-oxo-4-ethylbutyrolactone. Its ultraviolet spectrum in acid showed an absorption peak at 228 mμ which shifted to 262 mμ in base. Its infrared spectrum (KBr pellet) showed bands at 3.02, 5.75, 5.82, and 6.04 μ. Its n.m.r. spectrum[2] showed bands at τ 8.97 (3 H, triplet, J = 8 c.p.s., CH_3—CH_2), 8.22 and 8.37 (2 H, quartets, J = 6.5, 2 c.p.s., CH_3—CH_2—CH), 5.13 (1 H, sextet, J = 7, 2.5 c.p.s.; CH_2—CHOR—CH), 3.83 (1 H, doublet, J = 2 c.p.s. vinyl proton coupled with adjacent H), and 2.80 (1 H, enolic OH). The structure of III was conclusively established as 2-oxo-4-ethylbutyrolactone as follows: III was found to be identical (infrared spectrum, n.m.r. spectrum, and chromatographic behavior) with a sample of (±)-2-oxo-4-ethylbutyrolactone prepared by the published route from sodium diethyloxaloacetate and propionaldehyde.[3] A crystalline 2,4-dinitrophenylhydrazone of III was obtained, m.p. 168–171°, whose infrared and mass spectrum[4] were also found to be identical with those of a synthetic sample. However, the actual product of the enzymatic reaction is probably 2-oxo-4-hydroxycaproic acid (IV) rather than III, for III was not further metabolized by the cell-free extract. Also, III and IV have been shown to be readily interconvertible, depending on the hydrogen ion concentration of the surrounding medium.

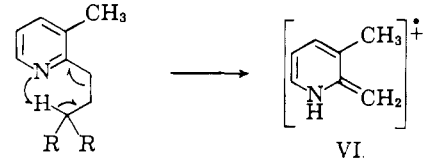
When the cell-free extract was treated with Sephadex G-25, a preparation was obtained which also readily metabolized I. For each mole of I present, 1 mole of O_2 was consumed and no CO_2 was evolved. During the reaction a yellow compound accumulated (λ_{max} 392 mμ (pH 13) and 315 mμ (pH 1.0)) which was slowly converted to II. This type of ultraviolet spectrum is reminiscent of those given by α-hydroxymuconic semialdehyde, α-hydroxy-γ-carboxymuconic semialdehyde, and 2,6-diketonon-4-enedioic acid.[5,6] Because of the extreme instability of this yellow intermediate, it was treated directly with NH_4OH to yield an amorphous pyridine compound[7] (V), m.p. 94–97°, λ_{max}^{alc} 273 mμ (ε 4700), λ_{max}^{KBr} 2.97, 5.78, and 5.86 μ; n.m.r. peaks at τ 8.82 (3 H, one tertiary CH_3), 7.52 (3 H, one CH_3 on pyridine ring), and 2.74 and 2.16 (2 H, aromatic protons).

Strong evidence for the assigned structure V was found in its mass spectrum. While there was no parent



V

ion at *m/e* 329, there was a prominent (M − CO_2) peak at *m/e* 285.[8] The base peak in the spectrum, *m/e* 107, is assigned to structure VI. The virtual absence of a



VI

peak at *m/e* 106 eliminates from consideration pyridines having the hydrindanedione substituent at C-3, C-4, or C-5,[9] since these should give rise to a prominent fragment VII.[10,11] Other prominent peaks at *m/e* 120 (VIII) and 55 (IX) also lend support to structure V.

(1) C. J. Sih, S. S. Lee, Y. Y. Tsong, and K. C. Wang, *J. Am. Chem. Soc.*, **87**, 1385 (1965).

(2) All n.m.r. spectra were determined on a Varian Associates recording spectrometer (A-60) at 60 Mc. in either deuterated chloroform or carbon tetrachloride. Chemical shifts are reported in τ-values (p.p.m.) [G.V.D. Tiers, *J. Phys. Chem.*, **62**, 1151 (1958)].

(3) A. Rossi and H. Schinz, *Helv. Chim. Acta*, 31, 473 (1948).

(4) The mass spectra of all samples were taken on a CEC-103 C mass spectrometer operating at 70-v. ionization voltage and employing a heated glass inlet system at 250°.

(5) S. Dagley, W. C. Evans, and D. W. Ribbons, *Nature*, **188**, 560 (1960).

(6) S. Dagley, P. J. Chapman, and D. T. Gibson, *Biochim. Biophys. Acta*, **78**, 781 (1963).

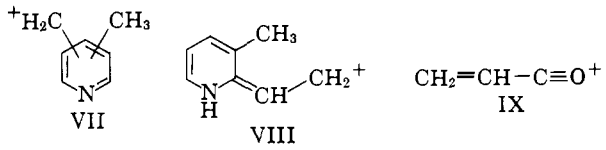
(7) The ring fission product of protocatechuic acid by the action of a 4,5-oxygenase was identified as α-hydroxy-γ-carboxymuconic semialdehyde. The latter compound was characterized by reaction with ammonia to yield 2,4-lutidinic acid (see ref. 5). Similarly, the ring fission product of β-phenylpropionate was characterized by an analogous reaction (see ref. 6).

(8) Decarboxylation of V probably occurred in the inlet system (250°).

(9) These would arise if oxidation of I occurred between C-2 and C-3.

(10) K. Biemann, "Mass Spectrometry, Organic Chemical Applications," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, pp. 132–135.

(11) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Interpretation of Mass Spectra of Organic Compounds," Holden-Day, Inc., San Francisco, Calif., 1964, pp. 253–256.

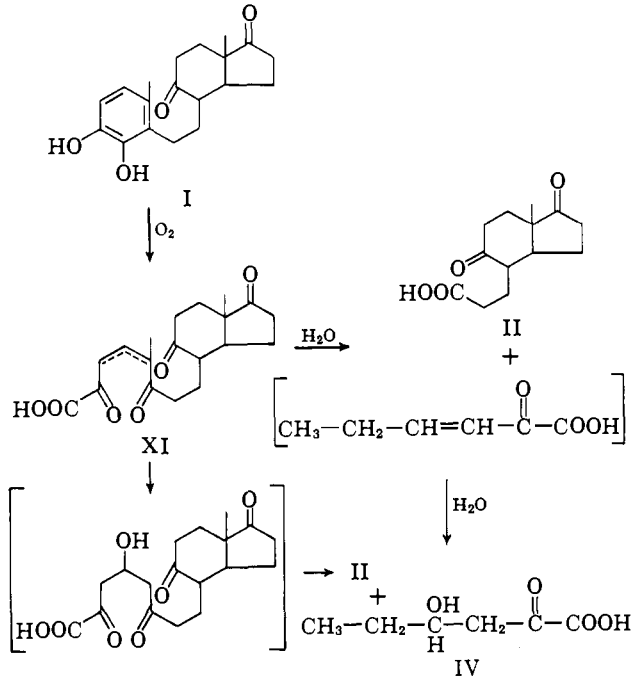


VII VIII IX

These results suggest that the structure of this yellow intermediate is 4(5),9(10)-diseco-3-hydroxyandrosta-1-(10),2-diene-5,9,17-trion-4-oic acid (XI). Treatment of XI with diazomethane afforded a methyl ester; its n.m.r. spectrum[12] showed a band at τ 6.16 characteristic of $COOCH_3$.

The participation of quinone in this aromatic ring fission reaction was eliminated by the following experiment. Incubation of I with mushroom tyrosinase afforded a quinone which readily condensed with [C^{14}]-ethylenediamine to give a radioactive dihydropyrazine compound.[13] When cell-free extracts of *N. restrictus* were substituted for mushroom tyrosinase in this reaction no dihydropyrazine derivative was noted. Furthermore, 9,10-secoandrosta-1,5(10)-diene-3,4,9,17-tetraone, prepared by oxidation of I with Ag_2O,[14] was very slowly converted in low yields to II when compared to I.

On the basis of these results it appears that the metabolism of I by *N. restrictus* involves oxidative fission between C-4 and C-5 of the aromatic A ring. Addition of water to the ethylenic bond followed by hydrolytic cleavage, or *vice versa*, affords II and 2-oxo-4-hydroxycaproic acid (IV), the latter compound being lactonized during the isolation procedure.[15]



(12) The n.m.r. spectrum of this methyl ester is complex because it consists of a mixture of keto–enol forms. However, the spectrum is consistent for the structure proposed.

(13) P. H. Jellinck and L. Irwin, *Biochim. Biophys. Acta*, **78**, 778 (1963).

(14) R. Willstätter and F. Müller, *Ber.*, **41**, 2581 (1908).

(15) This investigation was supported in part by research grants from the National Institutes of Health (AM 4874 and AM 6110).

(16) Wellcome Travel Fellow.

Charles J. Sih, K. C. Wang
D. T. Gibson,[16] H. W. Whitlock, Jr.
School of Pharmacy and Department of Chemistry
University of Wisconsin, Madison 6, Wisconsin
Received January 18, 1965

A New Route to Estrone from Sterols

Sir:

The initial degradative reactions of steroid hormones by microorganisms is reasonably well understood.[1-3] In contrast, the mechanism by which sterols are degraded in nature is not clearly defined. Turfitt[4] found that cholesterol was first oxidized to cholestenone and then to 3-ketoetiochol-4-enic acid and isocaproic acid by *Proactinomyces erythropolis*. A-Nor-3,5-seco-cholestan-5-on-3-oic acid was also identified as an intermediate in this conversion. Intact cells of a *Mycobacterium*[5] incubated with 4-C^{14}-cholesterol or 26-C^{14}-cholesterol oxidized carbon 4 of the ring to carbon dioxide about four times as rapidly as carbon 26 of the side chain. It was suggested that some compound or compounds retaining the side chain but with ring A opened may be intermediates.[6] On the other hand, in some microorganisms it has been shown that radioactive $C^{14}O_2$ appeared more rapidly from 26-C^{14}-cholesterol than 4-C^{14}-cholesterol.[7] Moreover, a *Nocardia* soil isolate in the presence of 8-hydroxyquinoline converted cholesterol in low yields to 3-ketobisnorchol-4-en-22-oic acid, 3-ketobisnorchola-1,4-dien-22-oic acid, androst-4-ene-3,17-dione, and androsta-1,4-diene-3,17-dione.[8] On the basis of these results it is not clear whether the side chain is cleaved prior to ring cleavage or *vice versa*. Thus, the attractive target of converting cholesterol into useful steroid intermediates by the microbial cleavage of the sterol side chain is still in its infancy.

It is well documented that one pathway of steroid degradation by microorganisms involves 1,2-dehydrogenation, followed by 9α-hydroxylation or *vice versa* with the rupture of the steroid ring B.[1] *Nocardia restrictus* (ATCC 14887) is an organism which degrades androst-4-ene-3,17-dione *via* this mechanism to yield 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione.[9] Since this organism is also capable of utilizing cholesterol as a sole carbon source, it appeared of interest to examine whether cholesterol is first degraded to C-19 steroids which are then metabolized by the conventional 9,10-seco pathway. When cholesterol was exposed to this organism, the only compound that one could isolate in reasonable yields was cholestenone; this product was then slowly metabolized in a series of reactions which eventually led to CO_2 and H_2O.

Previous studies have shown that 19-hydroxyandrost-4-ene-3,17-dione was rapidly converted into estrone by this organism[10] and the product, estrone, was not further metabolized. Figure 1 shows that 4-C^{14}-

(1) R. M. Dodson and R. D. Muir, *J. Am. Chem. Soc.*, **83**, 4627 (1961).

(2) C. J. Sih, S. S. Lee, Y. Y. Tsong, and K. C. Wang, *ibid.*, **87**, 1385 (1965).

(3) C. J. Sih, K. C. Wang, D. T. Gibson, and H. W. Whitlock, *ibid.*, **87**, 1386 (1965

(4) G. E. Turfitt, *Biochem. J.*, **42**, 376 (1948).

(5) T. C. Stadtman, A. Cherkes, and C. B. Anfinsen, *J. Biol. Chem.*, **206**, 511 (1954).

(6) It should be emphasized that even though $C^{14}O_2$ appeared more readily from 4-C^{14}-cholesterol than 26-C^{14}-cholesterol, this does not necessarily mean that the A ring is first ruptured prior to side-chain cleavage. It is quite conceivable that the side chain may be rapidly degraded, prior to ring fission, to a compound which in turn is slowly metabolized to $C^{14}O_2$.

(7) G. E. Peterson and J. R. Davis, *Steroids*, **4**, 677 (1964).

(8) J. M. Whitmarsh, 435th Meeting of the Biochemical Society, University of Leicester, England, Dec. 1963.

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

(10) C. J. Sih and A. M. Rahim, *J. Pharm. Sci.*, **52**, 1075 (1963).