IDENTIFICATION OF 2-OXO-CIS-4-HEXENOIC ACID AS A CLEAVAGE PRODUCT IN THE MICROBIOLOGICAL DEGRADATION OF STEROIDS

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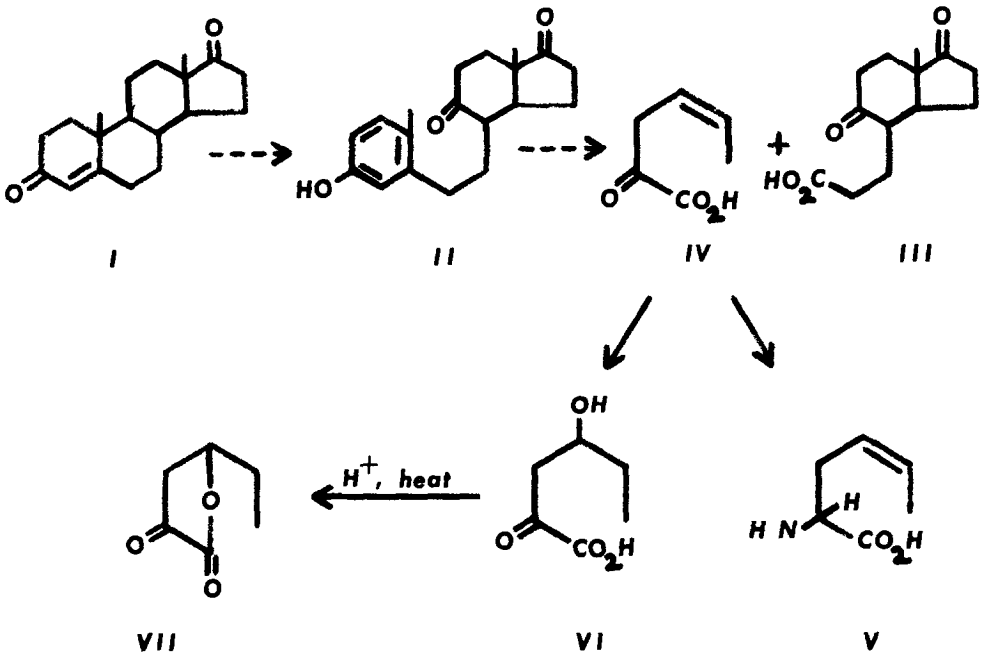
Received October 3, 1967

A number of microorganisms can effect the complete oxidative degradation of steroids by pathways which have been partially elucidated (see Talalay, 1965). Suitably supplemented cell-free extracts of Pseudomonas testosteroni rapidly oxidize Δ^4-androstene-3,17-dione-4-^{14}C (I) to $^{14}CO_2$, and under specified conditions, L-2-amino-cis-4-hexenoic acid-1-^{14}C (V) and DL-alanine 1-^{14}C (X) accumulate (Shaw et al., 1965). A partial pathway for the formation of these products is the following: Δ^4-androstene-3,17-dione (I) → $\Delta^{1,4}$-androstadiene-3,17-dione or 9α-hydroxy-Δ^4-androstene-3,17-dione → 3-hydroxy-9,10-seco-$\Delta^{1,3,5(10)}$-androsta-triene-9, 17-dione (II) → 7aß-methyl-1,5-dioxo-3aα-hexahydro-4-indanepropionic acid (III) and 2-oxo-cis-4-hexenoic acid (IV). The pathway to III is well documented (Dodson and Muir, 1961; Schubert et al., 1961; Gibson et al., 1966) and the enzymatic amination of IV could reasonably account for the accumulation of V (Shaw et al., 1965). Recently, 2-oxo-4-hydroxycaproic acid (VI) [isolated as 2-oxo-4-ethyl-butyrolactone (VII)] was obtained as a steroid degradation product of Nocardia restrictus, and VI



was shown to undergo reverse aldol cleavage to propionaldehyde (VIII) and pyruvate (IX) (Gibson et al., 1966), which by enzymatic amination may be converted to alanine (X) (Shaw et al., 1965).

In continuation of our studies on the enzymatic oxidation of steroids (Shaw et al., 1965), we now wish to report the synthesis of IV and present evidence for its participation in the degradation of ring A of Δ^4-androstene-3,17-dione (I) by P. testosteroni.

Condensation of 1-chloro-2-butyne with diethyl acetamidomalonate, gave diethyl 2-acetamido-2-carbethoxy-4-hexynoic acid in 91% yield, m.p. 75-77°, λ_{max}^{KBr} 3.05, 5.70, 6.05 and 6.50 μ; nmr ($CDCl_3$) signals at τ = 8.75 (6H, triplet), 8.28 (3H, triplet), 7.97 (3H, singlet), 6.84 (2H, quartet), 5.75 (4H, quartet), and 3.0 (1H, singlet). Hydrogenation of the triple bond in the presence of the deactivated catalyst (Lindlar, 1952) gave ethyl 2-acetamido-2-carbethoxy-cis-4-hexenoic acid (90% yield), m.p.

50-51°, λ_{max}^{KBr} 3.05, 5.70, 6.05, 6.55 and 14.25 μ; nmr ($CDCl_3$) signals at τ = 8.75 (6H, triplet), 8.40 (3H, doublet), 7.97 (3H, singlet), 6.90 (2H, doublet), 5.75 (4H, quartet), 4.2 - 5.1 (1H, multiplet), and 3.05 (1H, singlet). Basic hydrolysis of the latter followed by decarboxylation afforded DL-2-acetamido-cis-4-hexenoic acid in 78% yield, m.p. 114-116°, λ_{max}^{KBr} 3.0, 4.0-4.4, 5.20, 5.80, 6.25, 6.50 and 14.3 μ. Basic hydrolysis of the amide gave DL-2-amino-cis-4-hexenoic acid (V) (Skinner, Edson and Shive, 1961).

The cis and trans isomers of DL-2-amino-4-hexenoic acid and their hydrogenation product (norleucine) may be separated by chromatography on the amino acid analyzer column (Shaw et al., 1965). When synthetic V was analyzed by this method, less than 2% of the trans isomer and 1% of norleucine were detected. Synthetic V migrated identically on chromatography as the isolated amino acid (Shaw et al., 1965). Catalytic reduction of V at 25° and atmospheric pressure in the presence of palladized charcoal gave norleucine.

Treatment of V with trifluoroacetic anhydride in benzene yielded the N-trifluoroacetyl derivative (m.p. 89-91°), which was cyclized with N,N-dicyclohexylcarbodiimde in CH_2Cl_2 (Siemion and Nowak, 1960), to 2-trifluoromethyl-4-(2-cis-butenyl)-5-oxazolone, b.p. 88° (bath temp.)/0.25 mm, λ_{max}^{neat} 5.52, 6.05, 11.50 and 14.35 μ; nmr ($CDCl_3$) signals at τ = 8.70 (3H, doublet), 6.90 (2H, multiplet), 4.70 (2H, multiplet), and 4.10 (1H, multiplet). Hydrolysis of the oxazolone in phosphate-citrate buffer at pH 6.8 according to the method of Weygand, Steglich and Tanner (1962) afforded a compound with properties consistent with 2-oxo-cis-4-hexenoic acid (IV). The ketoacid (IV) (m.p. 62-92 with decomposition) could not be readily crystallized. The gummy crystals

gave an immediate blue-green color with $FeCl_3$. The IR spectrum ($CHCl_3$) showed peaks at 2.85, 3.7-4.2, 5.98, 6.02 (shoulder), 6.55, 6.95, 8.0, and 8.25 μ. The UV absorption maximum at 274 mμ shifted to 281 mμ in acid and 307 mμ in base. The nmr spectrum ($CDCl_3$) had peaks at τ = 8.4 (doublet), 8.15 (doublet), 4.2 (multiplet), 3.6 (triplet), and 4.35 (doublet). The accumulated data indicate that IV exists predominantly in the enol form. Catalytic hydrogenation of IV at 25° and atmospheric pressure gave 2-oxohexanoic acid, and treatment of IV with hot dilute sulfuric acid led to the known 2-oxo-4-ethylbutyrolactone (VII) ($\lambda_{max}^{H_2O}$ 228 mμ in acid, 262 mμ in base), described by Rossi and Schinz (1948).

Cell-free extracts of steroid-induced P. testosteroni converted IV-1-^{14}C (prepared from biosynthetic V-1-^{14}C) to V-1-^{14}C, X, and $^{14}CO_2$ in accordance with the proposed mechanism. The rapid enzymatic conversion of IV was followed by the disappearance of the absorption band at 274 mμ. During this conversion, no absorption at 228 mμ due to the formation of VII was observed. However, when dilute acid was added to the reaction system after disappearance of the 274 mμ absorption, followed by heating, the system developed the characteristic absorption of the lactone VII at 228 mμ, which shifted to 262 mμ in base. This lactonization occurred very much more readily than the conversion of IV to VII in hot dilute acid. Under identical conditions, similar cell-free extracts failed to metabolize the lactone VII, in confirmation of the report of Gibson et al. (1966).

These experiments support the view that IV is an intermediate in the degradative pathway of the steroid, and that it is hydrated to VI, rather than that hydroxylation of the steroid ring A at C-1 precedes cleavage of the 6-carbon fragment (Gibson et al., 1966). The lactone VII does not appear to be an intermediate in this pathway.

Acknowledgement. This work was supported by U.S. Public Health Service Research Grant AM 07422 and Training Grant GM 1183.

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