## The Microbiological Oxidation of Conessine

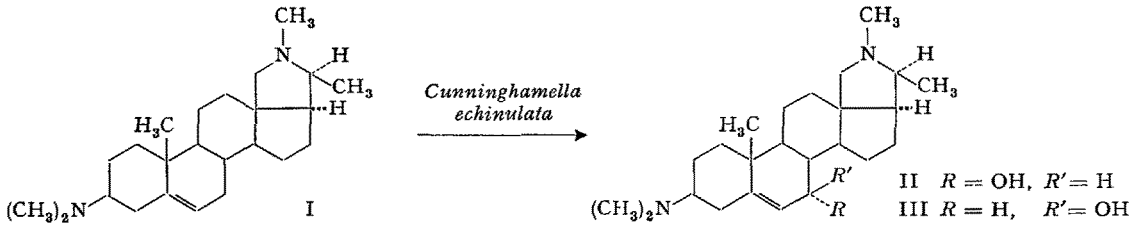
In the course of studies on the microbial transformation of alkaloids, it was observed that conessine (I) was converted by *Cunninghamella echinulata* into two more polar products. They were not readily separated by paper chromatography, but were resolved on alumina and identified as 7α-hydroxyconessine (II) and 7β-hydroxyconessine (III). After this work was completed, it was reported that *Aspergillus ochraceus* can also convert conessine to these products[1].

Evidence leading to the identification of the two products is as follows: By elemental analysis both II and III contained one more oxygen atom than conessine. The infrared absorption spectra of the two products in potassium bromide were similar and differed from that of conessine principally in having peaks in the regions of 3.05, 7.6 and 9.5 μ typical of alcoholic groups[2]. Thus the new oxygen function in both products was an alcohol. An acetate derivative formed with ease, suggesting the hydroxyl function in neither product was tertiary. Chromic acid oxidation in acetone converted both products into the same oxoconessine having one ultraviolet absorption maximum at 236 mμ ($\varepsilon = 13{,}200$), characteristic of an α,β-unsaturated carbonyl function.

The same conclusions were reached on the basis of molecular rotatory contributions.

Among the $\Delta^5$ steroids[5,6], the 7α-hydroxylated derivatives and their acetates show a more negative molecular rotation than the parent substance, whereas the corresponding epimeric 7β-derivatives have a more positive molecular rotation. Therefore, by this analogy as well, the C-7 hydroxyl was assigned the α-configuration in II and the β-configuration in III.

*Experimental. Cunninghamella echinulata* (NRRL A-11,498) was grown at 28° under conditions of agitation and aeration for a total of 130 h in 30 l of medium consisting of corn steep liquor, 2%; cerelose, 2%; ammonium sulfate, 1%; disodium acid phosphate, 0.6%; calcium carbonate, 0.5%; adjusted to pH 6.6. Conessine was added as a sterile solution in 0.1 *N* HCl to a final concentration of 0.2 mg/ml after 23 h of prefermentation. The harvest culture broth, adjusted to pH 8.7, was extracted twice with one volume of chloroform. Upon evaporation of the chloroform, the residue was dissolved in 100 ml of benzene and chromatographed on 200 g of aluminium oxide deactivated with 6% water, packed in a 3.2 cm-diameter column. The column was developed with 1100 ml of benzene/chloroform (1:1) which removed unchanged conessine. 7α-Hydroxyconessine (II) was eluted next with 900 ml of




The products were therefore epimers in which secondary hydroxyl groups were situated at either C-4 or C-7. The $\varepsilon_{max}$ value for the oxoconessine agreed more closely with those for the $\Delta^5$-7-ones ($\varepsilon = 8900$–$15{,}000$) than with the $\Delta^5$-4-ones ($\varepsilon = 3200$–$7200$) among the analogous α,β-unsaturated ketosteroids[3], indicating the carbonyl function was at C-7. Hence the two products were the epimeric 7-hydroxyconessines.

In the nuclear magnetic resonance spectra in deuterochloroform at 60 Mc, the C-6 proton signal was at 4.23 τ ($J = 6$ cps) in II and at 4.60 τ ($J = 1$ cps) in III. The resonance frequency of the C-7 proton occurred at 5.92 τ in II and at 6.04 τ in III. Since an equatorial proton absorbs at a lower frequency than its axial counterpart[4], the C-7 proton was assigned the equatorial configuration in II. The C-7 hydroxyl was therefore axial and α in II, and equatorial and β in III.

The molecular rotation ($M_D$) values of conessine and derivatives

| Compound | $M_D$ | $\Delta M_D$ |
|---|---|---|
| Conessine (I) | + 76 | – |
| 7α-Hydroxyconessine (II) | −160 | −236 |
| 7β-Hydroxyconessine (III) | +212 | +136 |
| 7α-Acetoxyconessine | −696 | −772 |
| 7β-Acetoxyconessine | +427 | +351 |
| 7-Oxoconessine | −152 | −228 |

chloroform, and finally 7β-hydroxyconessine (III) with 500 ml of chloroform/methanol (19:1). Alkaloidal material in the eluates was detected with Dragendorff reagent[7]. Composites of the eluate fractions containing the two products were evaporated to dryness and the residue from each was crystallized from acetone and methylene chloride/hexane (1:2). The yields were 544 mg of 7α-hydroxyconessine (II) and 281 mg of 7β-hydroxyconessine (III), or 11% and 5.6% respectively, based on the starting conessine.

7α-Hydroxyconessine (II), m.p. 171–172°, $[\alpha]_D^{25}$-43 (c, 0.63 in ethanol); Anal. calcd. for $C_{24}H_{40}N_2O$: C, 77.40; H, 10.74; N, 7.52. Found: C, 77.24; H, 11.13; N, 7.24. 7β-Hydroxyconessine (III), m.p. 206–208°, $[\alpha]_D^{25}$ + 57° (c, 0.80 in ethanol); Anal. C, 77.14; H, 10.90; N, 7.41[8].

[1] S. M. KUPCHAN, C. J. SIH, S. KUBOTA, and A. M. RAHIM, Tetrahedron Letters *26*, 1767 (1963).
[2] L. J. BELLAMY, *The Infra-red Spectra of Complex Molecules*, 2nd ed. (John Wiley and Sons Inc., New York 1958), p. 96.
[3] L. DORFMAN, Chem. Rev. *53*, 47 (1953).
[4] L. M. JACKMAN, *Nuclear Magnetic Resonance Spectroscopy* (Pergamon Press, New York 1959), p. 116.
[5] W. KLYNE, in *Determination of Organic Structures by Physical Methods* (E. A. BRAUDE and F. C. NACHOD ed., Academic Press Inc., New York 1955), vol. I, p. 73.
[6] J. P. MATHIEU and A. PETIT, *Constantes selectionnées, pouvoir rotatoire naturel, I. Stéroïdes* (Masson and Co., Paris 1956).
[7] H. THIES and F. W. REUTHER, Naturwiss. *41*, 230 (1954).
[8] The authors are indebted to Mr. L. BRANCONE for the microanalyses and to Mr. W. FULMOR for the NMR spectra.

7α-Acetoxyconessine was prepared by reacting 7α-hydroxyconessine with acetic anhydride/pyridine at room temperature overnight. The reaction product was purified by chromatography on alumina containing 6% water using ethyl ether as the eluting solvent, and crystallization from hexane/cyclohexane (2:1) and cyclohexane. Yield, 47%, m.p. 127.5–128.5°, $[\alpha]_D^{25}$ −168° (c, 0.92 in ethanol). Anal. calcd. for $C_{26}H_{42}N_2O_2$: C, 75.55; H, 10.01; N, 6.76. Found: C, 75.34; H, 10.31; N, 6.74.

7β-Acetoxyconessine was prepared from 7β-hydroxyconessine (III) in the same manner as described for 7α-acetoxyconessine. Yield, 58%, m.p. 173–174°, $[\alpha]_D^{25}$ +103° (c, 0.74 in ethanol). Anal. C, 75.46; H, 10.23; N, 7.09.

The oxidation of 7β-hydroxyconessine (III) with chromic acid in acetone[9] gave 7-oxoconessine. The reaction was quenched with aqueous methanol and the solution was made alkaline. The product was extracted into chloroform and crystallized from petroleum ether. Yield 59%, m.p. 158–159°, $[\alpha]_D^{25}$ −41° (c, 1.02 in ethanol). $\lambda_{max}^{CH_3OH}$ 236 mμ (ε = 13,200). Anal. calcd. for $C_{24}H_{38}N_2O$: C, 77.85; H, 10.26; N, 7.56. Found: C, 78.13; H, 10.08; N, 7.23.

A similar oxidation of 7α-hydroxyconessine (II) led to 7-oxoconessine, which was identified by ultraviolet and infrared absorption spectra and melting point.

*Zusammenfassung.* Conessin wurde mikrobiologisch durch *Cunninghamella echinulata* in zwei Produkte zerlegt, welche in reiner Form isoliert und mit 7α-Hydroxyconessin und 7β-Hydroxyconessin identifiziert werden konnten.

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[9] S. C. Pan, Anal. Chem. *34*, 766 (1962).

## Metabolic Properties of Micro-Organisms in Vibrating Culture

Earlier[1] we have reported that a vibrating stirrer has some distinct technical and biological advantages for obtaining submerged homogeneous growth of filamentous fungi. Among other properties it has been shown that a high rate of multiplication of *Aspergillus oryzae* can be obtained over a wide range of growth in the exponential phase. In stationary and shaken cultures this mould gives as high a multiplication rate only in the very early stages of growth (indirect estimation) when mycelial concentrations are very small and nutrients and oxygen freely available[2]. In later stages, where the mycelial content is measurable (above approximately 5 mg dry weight mycelium/100 ml) the highest rate of multiplication was about 0.175 duplications/h in substrate A[2] with shaken cultures, and about 0.095 duplications/h in the same substrate with stationary cultures, as compared with 0.3 in vibrating cultures.

In the linear phase of growth, which is part of the late exponential phase and part of the phase of decreasing rate of multiplication, the growth rates of *Aspergillus oryzae* are also considerably higher in Vibro Mix cultures than with other methods of cultivation; values of 25, 14.5 and 6.7 mg dry weight mycelium/100 ml h being obtained for Vibro Mix, shaken and stationary cultures respectively.

The apparatus proved to be a useful tool in some physiological investigations as reported below.

(1) *Influence of mechanical stress.* It is of some interest that cultures originating from different sizes of inocula react differently to mechanical stress. A small-inoculum culture ($4 \times 10^3$ conidia/100 ml) of *Aspergillus oryzae* grown with the Vibro Mixer gives a much lower yield in substrate A than does a large-inoculum culture ($2 \times 10^7$ conidia/100 ml), whereas stationary or shaken cultures do not show this effect if the same substrate is used (Figure 1). For the latter type of culture, on the other hand, it is characteristic that a somewhat smaller growth rate is obtained over a larger part of the growth curve when small inocula are used (see also Meyrath[2]). The larger the amplitude of the Vibro Mixer the stronger is the influence on maximum yield by inoculum size[3].

A major metabolic characteristic, which might be connected with the lower maximum yield of mycelium, is the marked excretion of organic nitrogenous compounds of small-inoculum cultures of *Aspergillus oryzae* when grown with the Vibro Mixer. The excretion is the more pronounced the larger the amplitude[3].

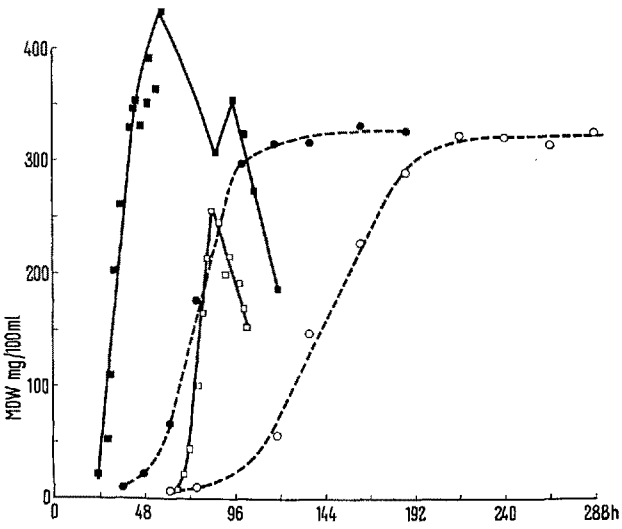


Fig. 1. Growth of *Aspergillus oryzae*, expressed in mg dry weight mycelium/100 ml, in Vibro Mix cultures (■, □) and in stationary cultures (●, ○) with large inocula (■, ●) and small inocula (□, ○).

[1] J. Meyrath, Exper. *20*, 235 (1964).
[2] J. Meyrath, Antonie van Leeuwenhoek, *29*, 57 (1963).
[3] A. F. McIntosh and J. Meyrath, J. gen. Microbiol. *33*, 57 (1963).