The 19-Hydroxylation Of Cortexolone By The Fungus *Pellicularia filamentosa*

A thesis presented in partial
fulfilment of the requirements for the degree
of Doctor of Philosophy
in Biotechnology at Massey University

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1982

ABSTRACT

The microbiological 19-hydroxylation of steroids was studied to investigate the feasibility of a microbial process to produce 19-hydroxysteroids. These are important precursors of the valuable 19-norsteroids. At present, in industrial processes, 19-hydroxylation is performed by chemical synthesis.

Fungi, selected from the genera *Pestalotia* (25 strains) and *Pellicularia* (5 strains), were screened for their steroid-hydroxylating activities. Thus, hydroxylation of the substrates progesterone, 4-androstene-3,17-dione, and cortexolone (17α,21-dihydroxy-4-pregnene-3,20-dione) was studied. Of the organisms tested, only *Pellicularia filamentosa* f.sp. *microsclerotia* IFO 6298 and *Pellicularia filamentosa* f.sp. *sasakii* IFO 5254 were found to perform 19-hydroxylation. Thus, both fungi could produce 19-hydroxycortexolone from cortexolone, with the former organism the more active in this respect. Hydrocortisone (11β-hydroxycortexolone) was also produced by both organisms. Neither organism, however, could similarly hydroxylate progesterone nor 4-androstene-3,17-dione. With these substrates, products other than the 19-hydroxylated derivatives were formed. Thus a degree of substrate specificity was recognised for steroidal-19-hydroxylation by these fungi. None of the *Pestalotia* species tested could 19-hydroxylate any of the three substrates, despite claims in the literature, but instead were very active in 11α-hydroxylation. In particular, many species were able to 11α-hydroxylate progesterone (0.5 g/l concentration) in greater than 90% ($^{w}/_{w}$) yield.

Using *P. filamentosa* f.sp. *microsclerotia* IFO 6298, in batch fermentation, at a cortexolone concentration of 0.5 g/l yields of 19- and 11β-hydroxycortexolone totalled approximately 40% ($^{w}/_{w}$) of

the consumed substrate. The ratio of the two products, typically, was approximately 1.2:1 (19:11β). Only small variations in this ratio were ever observed. The steroid losses which were observed did not proceed via the hydroxy products as intermediates, but via a degradation pathway, from cortexolone, parallel to the hydroxylation reactions.

The 11β- and 19-hydroxylase enzyme-system of *P. filamentosa* f.sp. *microsclerotia* IFO 6298 was shown to be inducible by cortexolone. By using the protein synthesis inhibitor, cycloheximide, in fermenter culture the effects of dissolved oxygen tension (DOT) on enzyme induction and enzyme expression were separately investigated. For both hydroxylations, an optimum DOT for induction was shown at 15% of saturation, while the optimum for expression is at 30% of saturation. Thus, maximum rates of hydroxylation were achieved when induction was performed at low DOT, followed by elevation to ensure maximum expression.

The effects of specific glucose consumption rate and specific growth rate were investigated using chemostat cultures, under automatic DOT control (at 30% of saturation). At a constant specific growth rate, the importance of glucose metabolism to the hydroxylation process was demonstrated. Thus, with glucose-limited cultures, decreasing specific hydroxylation rates were observed with decreasing specific glucose consumption rates, possibly as a result of the restricted availability of NADPH, which is required for hydroxylation to occur. Conversely, with nitrogen-limited cultures, it was observed that the hydroxylase system is subject to glucose repression. Thus, with high specific glucose consumption rates, cultures showed low levels of hydroxylation activity. Maximum activity was obtained at a point, which apparently represents a balance between sufficient glucose metabolism to

maintain full expression of activity and a condition of excess glucose uptake resulting in repression of activity. This finding may be of considerable significance for fungal steroid-hydroxylation processes in general, since relief of repression, when it exists, could give several-fold increases in specific hydroxylation rates as observed in this study. Over the range of specific growth rates studied (0.028 h^{-1} to 0.119 h^{-1}) no significant effect on specific hydroxylation rates was observed.

ACKNOWLEDGEMENTS

The author wishes to acknowledge the following people:

Drs I.S. Maddox and R. Chong for guidance and supervision. Their availability and willingness to discuss problems, as they arose, was greatly appreciated.

Professor R.L. Earle, Head of the Biotechnology Department, for continual encouragement over the course of the project.

Professor R. Hodges of the Department of Chemistry, Biochemistry, and Biophysics, Massey University and Dr J. Shaw of the Applied Biochemistry Division, D.S.I.R., Palmerston North for performing Mass Spectral Characterisation of compounds. I particularly wish to thank Prof. Hodges for the many helpful discussions concerning this part of my work.

Dr K. Jolley, of the Department of Chemistry, Biochemistry and Biophysics, Massey University for performing NMR measurements and for discussing the results.

Mr T. Hesketh, of the Industrial Management and Engineering Department, Massey University for designing the DOT control circuit and for assistance with analysis of the system.

Mr T. Seddon, of the above department, for his patient assistance with the construction and commissioning of the DOT control system.

Dr P. Austin, also of the above department, for the many helpful discussions concerning the DOT controller.

Mr D. Couling, Mr J. Alger, and Mr P. Shaw, of the Biotechnology Department, Massey University, for assistance with the many technical problems which arose concerning equipment.

Mr M. Stevens, Mrs R. Caulder, Mr M. Lubbers, and Mrs A. McCutcheon, of the above department, for their excellent laboratory support.

Mr P. Ellingham for drawing of chemical structures.

Mrs P. Fleet for her excellent typing of this thesis.

Karen for proof-reading of the thesis, figure head-lining, and for her love and encouragement.

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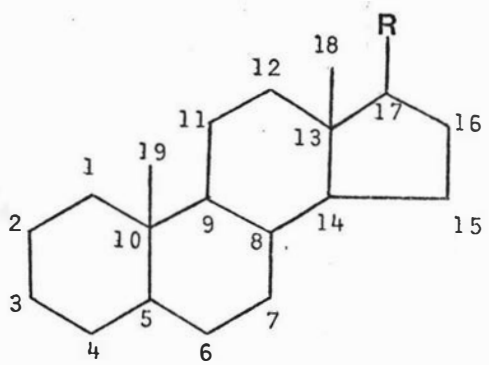
STEROID NOMENCLATURE AND STRUCTURE

The trivial names used for steroids are given, followed by the abbreviations (if any) employed, in brackets, and their systematic chemical names (I.U.P.A.C.-I.U.B., 1969).

Numbering of the steroid ring system and side chain groups is as follows:



R	Stem Name
-H	androstane
20 21 $-CH_2-CH_3$	pregnane

Androstenedione (AD) = 4-androstene-3,17-dione

Estrone = 3β-hydroxy-19-nor-1,3,5(10)-androstatrien-17-one

Cortexolone (Co) = 17α,21-dihydroxy-4-pregnene-3,20-dione

Hydrocortisone = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione

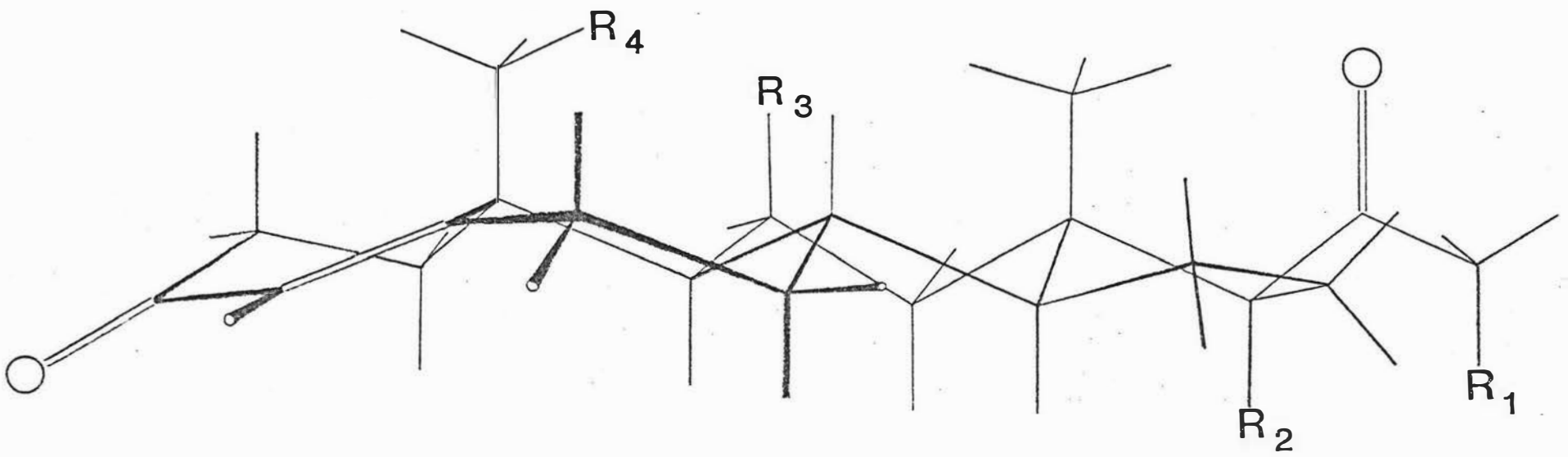
Norethisterone = 17α-ethynyl-17β-hydroxy-19-nor-4-androsten-3-one

Oestradiol = 3β,17β-dihydroxy-19-nor-1,3,5(10)-androstatriene

Progesterone (Pr) = 4-pregnene-3,20-dione

Pregnenolone Acetate = 3β-hydroxy-5-pregnen-20-one acetate

The following figure shows a Dreiding Model of substituted progesterone. The axial and equatorial nature of the various substituent bonds, around the ring system, can be clearly seen.



Dreiding Model of Substituted Progesterone

$R_1 = R_2 = R_4 = OH$, $R_3 = H$ 19-hydroxycortexolone

$R_1 = R_2 = R_3 = OH$, $R_4 = H$ 11β-hydroxycortexolone

DESIGNATION OF STERIOCHEMISTRY

α	(alpha)	designates a bond below the plane of the ring system
β	(beta)	designates a bond above the plane of the ring system
ξ	(xi)	designates a bond of unknown steriochemistry

ABBREVIATIONS

Abbreviations of Units

amu	atomic mass unit
^{o}C	degrees Celsius
d	day
g	gram
h	hour
l	litre
m	meter
M	mole/litre
mM	millimole/litre
m/e	mass:charge ratio
min	minute
mg	milligram
ml	millilitre
nm	nanometer
psi	pounds per square inch
rpm	revolutions per minute
s	second
µF	micro Farad
Ω	Ohm
KΩ	Kiloohm

Other Abbreviations

AcO-	acetyl
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
An.	Acetone
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUFS	Absorbance Units Full Scale
B.P.	Boiling Point
C	Capacitor
cAMP	cyclic AMP
Cyt-P_{450}	Cytochrome-P_{450}
D	Dilution Rate (h^{-1})
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
DOT	Dissolved Oxygen Tension
DW	Dry Weight (g/l)
EtOH	Ethanol
HOAC	Acetic Acid
HOBr	Hypobromous Acid
hplc	High Performance Liquid Chromatography
I.D.	Internal Diameter
IFO	Institute for Fermentation, Osaka
IMI	Commonwealth Mycological Institute
IPA	Isopropyl alcohol
I.R.	Infra-red
MDW	Mycelial Dry Weight (g/l)
MeOH	Methanol
MNC	Mycelial Nitrogen Concentration (g/l)

m.p.	Melting Point
MPN	Mycelial Percentage Nitrogen (% w/w)
mRNA	messenger RNA
m.s.	Mass Spectrum
N	Nitrogen
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NBA	N-Bromoacetamide
NMR	Nuclear Magnetic Resonance
PDDCC	Plant Diseases Division Culture Collection (N.Z. D.S.I.R.)
pTsOH	para-Toluene sulphonic acid
Py	Pyridine
q_{19}	Specific Rate of 19-Hydroxylation (mg/g N.h)
$q_{11\beta}$	Specific Rate of 11β-Hydroxylation (mg/g N.h)
q_{gl}	Specific Glucose Consumption Rate (g/g N.h) (g/g DW.h)
R	Resistor
R_f	Tlc mobility of a compound relative to the solvent front mobility
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
R_t	Hplc Retention time (min)
S	Glucose concentration in the fermenter (g/l)
S_o	Feed-medium glucose concentration (g/l)
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
tlc	Thin Layer Chromatography
UV	Ultra-violet
μ	Specific Growth Rate (h^{-1})