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THE 19-HYDROXYLATION OF
CORTEXOLONE BY THE FUNGUS
Pellicularia filamentosa

A thesis presented in partial
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of Doctor of Philosophy
in Biotechnology at Massey University

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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 (17 α , 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 (11 β -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 11 α -hydroxylation. In particular, many species were able to 11 α -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 11 β -hydroxycortexolone totalled approximately 40% (w/w) of

the consumed substrate. The ratio of the two products, typically, was approximately 1.2:1 (19:11 β). 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 11 β - 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.

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TABLE OF CONTENTS

v
PAGE

Abstract	i
Acknowledgements	iv
Table of Contents	v
List of Figures	x
List of Tables	xv
Steroid Nomenclature and Structure	xvii
Abbreviations	xx
<u>CHAPTER 1</u> Introduction	1
<u>CHAPTER 2</u> Literature Survey	3
2.1. Introduction	3
2.2 Microbiological 19-Hydroxylation of Steroids	6
2.3 Microbiological Steroid Hydroxylases	17
2.3.1 Mechanisms of Hydroxylation	18
2.3.2 Hydroxylase Biochemistry	23
2.4 Fermentation Technology, Applied to Steroid Hydroxylation by Fungi	32
<u>CHAPTER 3</u> Materials and Methods	35
3.1 Materials	
3.1.1 Microbiological Media	35
3.1.2 Chromatographic Materials	35
3.1.3 Gases	35
3.1.4 Chemicals	35
3.1.4.1 Steroids	35
3.1.4.2 Solvents	45
3.1.4.3 Other Chemicals	45
3.1.5 Organisms	47
3.2 Media Sterilization	48
3.3 Cleaning of Glassware	48
3.4 Analytical Methods	49
3.4.1 Melting Points	49
3.4.2 pH Measurement	49
3.4.3 Mycelial Dry Weight Determination	49

	<u>PAGE</u>	
3.4.4	Glucose Analysis	49
3.4.5	Total Carbohydrate Analysis	49
3.4.6	Total Nitrogen Determination	50
3.4.7	Thin Layer Chromatography (tlc)	50
3.4.8	High Performance Liquid Chromatography (hplc)	51
3.4.9	Infra-red Spectrophotometry (IR)	52
3.4.10	Ultra-violet Spectrophotometry (UV)	54
3.4.11	Mass Spectrometry (MS)	54
3.4.12	Nuclear Magnetic Resonance Spectroscopy (NMR)	54
3.5	Culture Conditions	54
3.5.1	Shake-Flask Culture	54
3.5.2	Fermenter Culture	56
3.5.2.1	Equipment, Instrumentation and Control	56
3.5.2.2	Continuous Culture Operation	64
3.5.2.3	Sterilization	66
3.5.2.4	Inoculum Preparation	66
3.5.2.5	Steroid Addition	66
3.5.2.6	Fermenter Operation	67
3.6	Steroid Extraction and Analytical Sample Preparation	67
3.6.1	Extraction of Freeze-Dried Cultures	68
3.6.2	Solvent Extraction	69
3.7	Chemical Methods Used For Compound Identification	70
3.7.1	Acetylation	70
3.7.2	Chromium Trioxide Oxidation	70
3.7.3	Dehydration of 16 α -Hydroxyprogesterone	71
3.7.4	Sodium Bismuthate Oxidation	71
3.8	Calculations	71
3.8.1	Hplc Data Analysis	71
3.8.2	Dilution Corrections for Fermenter Transformations	72
3.9	Discussion of Methods	75
3.9.1	Nuclear Magnetic Resonance Spectroscopy (NMR)	75
3.9.2	Mass Spectrometry of C-19-Hydroxysteroids	77
3.9.3	Analytical Methods	78

PAGE

<u>CHAPTER 4</u>	Examination of Some Steroid-Transforming Abilities of the Genera <i>Pestalotia</i> (<i>sensu</i> Guba) and <i>Pellicularia</i> (<i>sensu</i> Rodgers)	80
4.1	Introduction	80
4.2	Taxonomy of the Genera <i>Pellicularia</i> and <i>Pestalotia</i>	80
4.2.1	<i>Pellicularia</i> (<i>sensu</i> Rodgers)	80
4.2.2	<i>Pestalotia</i> (<i>sensu</i> Guba)	81
4.3	Experimental Methods	83
4.4	Results and Discussion	84
4.4.1	The Transformation of Progesterone by <i>Pestalotia</i> Species	84
4.4.2	The Transformation of 4-Androstene-3,17-dione by <i>Pestalotia</i> Species	86
4.4.3	The Transformation of Cortexolone by <i>Pestalotia</i> Species	88
4.4.4	The Transformation of Cortexolone by <i>Pellicularia</i> Species	90
4.4.4.1	<i>P. filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298	91
4.4.4.2	<i>P. filamentosa</i> f.sp. <i>sasakii</i> IFO 5254	93
4.4.4.3	<i>Corticium practicola</i> IFO 6253	93
4.4.4.4	<i>Corticium caeruleum</i> IFO 4974 and <i>P. filamentosa</i> f.sp. <i>solani</i> IFO 5289	96
4.4.5	The Transformation of 4-Androstene-3,17-dione and Progesterone by <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298 and <i>sasakii</i> IFO 5254	96
4.5	Conclusions	100
<u>CHAPTER 5</u>	Preliminary Studies on the 11 β - and 19-Hydroxylation of Cortexolone by <i>Pellicularia filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298	104
5.1	Introduction	104
5.2	The Time Course of Cortexolone Hydroxylation in Fermenter Culture	104
5.2.1	Introduction	104
5.2.2	Results and Discussion	104
5.3	The Effect of Growth Medium on the 11 β - and 19-Hydroxylation of Cortexolone	110
5.3.1	Introduction	110
5.3.2	Results and Discussion	111

	<u>PAGE</u>
5.4 The Effect of Culture pH on the 11 β - and 19-Hydroxylation of Cortexolone	111
5.4.1 Introduction	111
5.4.2 Results and Discussion	113
5.5 The Effect of Method of Substrate Addition on the 11 β - and 19-Hydroxylation of Cortexolone	115
5.5.1 Introduction	115
5.5.2 Results and Discussion	115
5.6 The Inducibility of the 11 β - and 19-Hydroxylase System of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i>	117
5.6.1 Introduction	117
5.6.2 Results and Discussion	117
5.7 Binding of Cortexolone and 11 β -Hydroxycortexolone by Mycelia	120
5.7.1 Introduction	120
5.7.2 Results and Discussion	121
5.8 The Effects of α,α' -Bipyridyl and 8-Hydroxyquinoline on the 11 β - and 19-Hydroxylation of Cortexolone	122
5.8.1 Introduction	122
5.8.2 Results and Discussion	122
5.9 The Effects of Phenobarbital and Phenanthrene on the 11 β - and 19-Hydroxylation of Cortexolone	124
5.9.1 Introduction	124
5.9.2 Results and Discussion	124
5.10 The Stability of Hydroxylated Cortexolone in <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> Cultures, and the Possibility of Product Inhibition of the Hydroxylation Process	126
5.10.1 Introduction	126
5.10.2 Results and Discussion	127
5.11 Conclusions	129
<u>CHAPTER 6</u> The Effect of Dissolved Oxygen Tension on the Induction and Expression of the Cortexolone Hydroxylase System of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i>	131
6.1 Introduction	131
6.2 Experimental Method	131
6.3 Results	133
6.4 Discussion	139
6.5 Conclusions	146

	<u>PAGE</u>
<u>CHAPTER 7</u> The Effect of Glucose Consumption Rate and Growth Rate on the 11 β - and 19-Hydroxylation of Cortexolone by <i>P. filamentosa</i> f.sp. <i>microsclerotia</i>	147
7.1 Introduction	147
7.2 Batch and Fed-Batch Experiments	148
7.3 Chemostat Fermentations	154
7.3.1 Introduction	154
7.3.2 Experimental Method	155
7.3.3 Results and Discussion	156
7.3.4 Conclusions	178
 <u>CHAPTER 8</u> Final Discussion and Conclusions	 180
 References	 184
 <u>APPENDIX A</u> The Dissolved Oxygen Tension Controller	 197
A.1 Introduction	197
A.2 The Equipment	197
A.3 Controller Tuning	203
 <u>APPENDIX B</u> Chemical Spectra	 213
 <u>APPENDIX C</u> Multiple Linear Regression Analysis of Specific Rate of 19-Hydroxylation Versus Specific Growth Rate and Mycelial Percentage Nitrogen	 228
 <u>APPENDIX D</u> Reprint of Publication Concerning Work Described in this Thesis	 230

LIST OF FIGURESPAGE

2.1	A typical synthetic route for the production of 19-hydroxysteroids.	4
2.2	The 19-hydroxylation of cortexolone by <i>Corticium sasakii</i> .	5
2.3	The action of <i>C. decora</i> on 5 α -androstan-17-one (Chambers <i>et al.</i> , 1975).	13
2.4	The microbial transformation of 1,4,7-androstatriene-3,17-dione (I) to equilin (II) and equilenin (III).	15
2.5	A diagram showing the hypothesised similarity in spatial relationship between enzymic hydroxylation and epoxidation (Hayano, 1962).	20
2.6	A possible mechanism for steroid hydroxylations at 6 β and 21 positions.	22
2.7	A model for steroid hydroxylation via Cytochrome-P ₄₅₀ -linked monooxygenases.	24
2.8	Sites within mammalian cells that are reportedly important in the induction of Cytochrome-P ₄₅₀ monooxygenases.	27
3.1	Synthesis of 19-hydroxyprogesterone.	38
3.2	Synthesis of 16 α -hydroxyprogesterone.	41
3.3	Synthesis of 11 α -hydroxy-4-androstene-3,17-dione.	43
3.4	The hplc separation of some monohydroxy derivatives of progesterone from progesterone (Pr) and androstenedione (AD).	53
3.5	The hplc separation of some monohydroxy derivatives of androstenedione from androstenedione (AD)	53
3.6	The hplc separation of monohydroxy derivatives of cortexolone from cortexolone (Co) and 11 α -hydroxyprogesterone (11 α -OH-Pr).	53
3.7	A schematic diagram showing the fermenter vessel and placement of the various facilities and probes in the vessel head.	57
3.8	A schematic diagram of the fermenter plus its ancilliary equipment (in continuous-culture operation).	58
3.9	The fermenter and ancilliary equipment (in continuous-culture operation)	59
3.10	A schematic diagram of the DOT control system which utilized on-off control of aeration rate.	63

	<u>PAGE</u>
3.11 A schematic diagram of the DOT control system which utilized proportional-integral control of agitation speed.	63
3.12 A schematic diagram of continuous culture overflow and medium feeding facilities.	65
3.13 Peak heights versus quantity injected, for the hplc analysis of cortexolone, 11 α -hydroxyprogesterone 11 β -hydroxycortexolone, and 19-hydroxycortexolone.	79
4.1 Hplc chromatographs of the culture extracts of the indicated fungi (after 8 h incubation with androstenedione) and of a standard mixture containing androstenedione, 11 α -, 11 β -, and 19-hydroxyandrostenedione.	99
4.2 The synthesis of 17 α -ethynyl-19-nortestosterone (19-norethisterone) from 19-hydroxyandrostenedione.	102
4.3 The synthesis of 19-hydroxyandrostenedione from 19-hydroxycortexolone.	102
5.1 The time course for the hydroxylation of cortexolone by a fermenter culture of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298.	106
5.2 The time course for the hydroxylation of cortexolone by a fermenter culture of <i>P. filamentosa</i> f.sp. <i>sasakii</i> IFO 5254.	107
5.3 The time course for the hydroxylation of cortexolone by a fermenter culture of <i>Corticium practicola</i> IFO 6253.	108
5.4 The course of cortexolone hydroxylation by a fermenter culture of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> , in the presence of cycloheximide (250 μ g/ml).	119
6.1 The effect of DOT on the specific rate of expression of the cortexolone-11 β - and 19-hydroxylation activities of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> .	135
6.2 The effect of DOT on the induction of the cortexolone-11 β - and 19-hydroxylation activities of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> .	135
6.3 The initial time course for the hydroxylation of cortexolone by <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> under DOT control at 30% of saturation.	137
6.4 The initial time course for the hydroxylation of cortexolone by <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> , under DOT control at 15% of saturation (for the first 4 h), and then, 30% of saturation.	138

	<u>PAGE</u>	
6.5	Selected examples of the course of hydroxylase <u>expression</u> , under different DOT conditions, during the 2 h period after cycloheximide addition (DOT during induction was 25% of saturation).	140
6.6	Selected examples of the course of hydroxylation, during the 2 h period after cycloheximide addition, following induction under different DOT conditions (DOT during expression was 30% of saturation).	140
6.7	The determination of a $(DOT)_{crit}$ value for <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> .	
	(a) By following the oxygen consumption rate as DOT declined during growth.	
	(b) By measuring oxygen-exhaustion curves, with air in the fermenter head space and nitrogen in the fermenter head space.	144
7.1	The course of cortexolone hydroxylation by a fermenter culture (initial cortexolone concentration, 2.5 g/l).	150
7.2	The course of cortexolone hydroxylation by a fermenter culture which was periodically fed with glucose.	151
7.3	The effect of glucose-exhaustion, and subsequent glucose feeding, on the course of cortexolone hydroxylation.	152
7.4	The course of cortexolone hydroxylation by a culture which was continuously fed with glucose and cortexolone.	153
7.5	Effect of dilution rate on the specific rates of cortexolone hydroxylation.	157
7.6	Effect of feed-medium glucose concentration on the specific rates of cortexolone hydroxylation at $D = 0.064 \text{ h}^{-1}$.	160
7.7	Plots of q_{19} versus q_{g1} , expressed	
	(a) in terms of mycelial dry weight, and	
	(b) in terms of mycelial nitrogen.	162
7.8	Time course of cortexolone hydroxylation, with a "step" change in glucose consumption rate at 8 h.	164
7.9	Scheme depicting the action of cyclic AMP on the <u>lac</u> operon (after Pastan and Perlman, 1970).	166
7.10	Effect of feed-medium glucose concentration on the specific rates of cortexolone hydroxylation at $D = 0.028 \text{ h}^{-1}$.	168
7.11	Effect of feed-medium glucose concentration on the specific rates of cortexolone hydroxylation at $D = 0.119 \text{ h}^{-1}$.	170
7.12	Specific glucose consumption rate for optimum hydroxylation rates, $(q_{g1})_{op}$, versus dilution rate and specific growth rate.	172

	<u>PAGE</u>
7.13 Mycelial nitrogen concentration and mycelial percentage nitrogen versus feed medium glucose concentration.	174
7.14 Mycelial percentage nitrogen versus specific glucose consumption rate, (a) in terms of mycelial nitrogen, and (b) in terms of mycelial dry weight.	175
7.15 Specific rate of 19-hydroxylation versus mycelial percentage nitrogen.	177

APPENDICES

A1 The control loop.	198
A2 A circuit diagram of the equipment.	200
A3 Circuit diagram of control-board power supply.	202
A4 The calibration characteristic of the thyristor motor-drive unit i.e., the relationship between agitator speed and the voltage output of the PI-controller.	205
A5 The PI-controller circuit.	203
A6 The "step-response" behaviour of the PI-controller.	204
A7 The response of the DOT probe to a step change from deoxygenated water to air-saturated water.	205
A8 The closed-loop block diagram.	207
A9 A typical process reaction curve, showing the methods for determining ΔR , T_d , and L.	208
A10 The root locus diagram for the system's open-loop transfer function.	211
A11 Typical performance characteristics of the control system. (a) The DOT response to step changes in set-point value. (b) A typical DOT-trace during fermenter operation.	212
B1 Mass spectrum of synthesised 19-hydroxyprogesterone.	214
B2 Mass spectrum of synthesised 16 α -hydroxyprogesterone.	215
B3 Mass spectrum of 19-hydroxycortexolone; product of the action of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298 on cortexolone.	216
B4 Mass spectrum of 19-hydroxy-4-androstene-3,17-dione; prepared by sodium bismuthate degradation of 19-hydroxycortexolone (product of the action of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298 on cortexolone).	217

		<u>PAGE</u>
B5	IR spectral characterisation of the progesterone hydroxylation product of <i>Pestalotia</i> sp. PDDCC 3062.	218
B6	Mass spectrum of cortexolone.	219
B7	IR spectral characterisation of putative 11 β -hydroxy-cortexolone; product of the action of <i>P. filamentosa</i> f.sp. <i>sasakii</i> IFO 5254 on cortexolone.	220
B8	Mass spectrum of 19-hydroxycortexolone; product of the action of <i>P. filamentosa</i> f.sp. <i>sasakii</i> IFO 5254 on cortexolone.	221
B9	Mass spectrum of putative 6 β -hydroxycortexolone; product of the action of <i>C. practicola</i> IFO 6253 on cortexolone.	222
B10	Mass spectrum of putative 14 α -hydroxy-4-androstene-3,11,17-trione; product of the action of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298 on androstenedione.	223

LIST OF TABLES

	<u>PAGE</u>
2.1 Patent claims for microbiological 19-hydroxylation of steroids	7
2.2 References from the Scientific literature for microbiological 19-hydroxylation of steroids.	8
2.3 The transformation of C ₁₉ steroids by <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298.	10
3.1 Media composition.	36
3.2 Important signals in the ¹ H-NMR spectra of various steroids	75
3.3 Chemical shift data for the ¹³ C-NMR spectra of various steroids.	76
4.1 The action of <i>Pestalotia</i> species on progesterone.	85
4.2 The action of <i>Pestalotia</i> species on androstenedione.	87
4.3 The action of <i>Pestalotia</i> species on cortexolone.	89
4.4 The action of various <i>Pellicularia</i> (<i>sensu</i> Rodgers) species on cortexolone.	97
5.1 The 11 β - and 19-hydroxylation of cortexolone by <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> in different media.	112
5.2 The effect of medium pH on the transformation of cortexolone.	114
5.3 The effect of different "carrying solvents" on the cortexolone transformation.	116
5.4 The effect of cycloheximide on the 11 β - and 19-hydroxylation of cortexolone.	117
5.5 Recoveries for cortexolone and 11 β -hydroxycortexolone after incubation with mycelia.	121
5.6 The effect of 8-hydroxyquinoline and α,α' -bipyridyl on the 11 β - and 19-hydroxylation of cortexolone.	123
5.7 The effect of phenobarbital and phenanthrene on the 11 β - and 19-hydroxylation of cortexolone.	125
5.8 Steroid concentrations in shake-flask cultures, after 24 h incubation.	128
6.1 The effect of cortexolone concentration on enzyme induction.	133
6.2 The effect of DOT on the rates of cortexolone hydroxylation by cultures induced at a DOT value of 50% of saturation.	136

	<u>PAGE</u>	
6.3	Comparison of hydroxylation rates from fermentations performed in the presence and absence of cycloheximide.	139
7.1	The complete experimental data set for Figure 7.5.	158
7.2	The complete experimental data set for Figure 7.6.	161
7.3	The complete experimental data set for Figure 7.10.	169
7.4	The complete experimental data set for Figure 7.11.	171

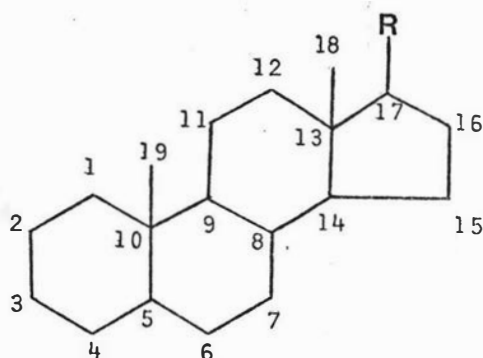
APPENDICES

B1	Proton and Carbon-13 Nuclear Magnetic Resonance spectra of progesterone and synthesised 19-hydroxyprogesterone.	224
B2	Proton and Carbon-13 Nuclear Magnetic Resonance spectra of 19-hydroxycortexolone (product of the action of <i>P. filamentosa</i> f.sp. <i>microsclerotia</i> IFO 6298 on cortexolone).	225
B3	Carbon-13 Nuclear Magnetic Resonance spectrum of 11 α -hydroxyprogesterone (product of the action of <i>Pestalotia</i> sp. PDDCC 3062 on progesterone).	226
B4	Carbon-13 Nuclear Magnetic Resonance spectrum of putative 6 β -hydroxycortexolone (product of the action of <i>Corticium praticola</i> IFO 6253 on cortexolone).	227

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).

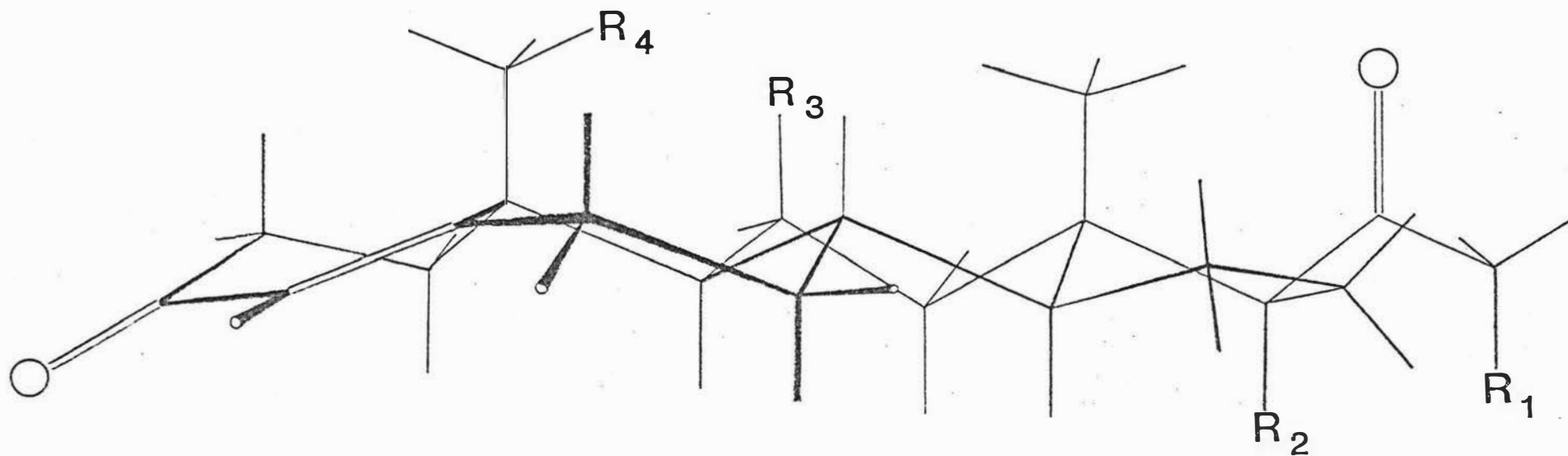
Numbering of the steroid ring system and side chain groups is as follows:



R	Stem Name
-H	androstane
20 21 -CH ₂ -CH ₃	pregnane

Androstenedione (AD)	=	4-androstene-3,17-dione
Estrone	=	3 β -hydroxy-19-nor-1,3,5(10)-androstatrien-17-one
Cortexolone (Co)	=	17 α ,21-dihydroxy-4-pregnene-3,20-dione
Hydrocortisone	=	11 β ,17 α ,21-trihydroxy-4-pregnene-3,20-dione
Norethisterone	=	17 α -ethynyl-17 β -hydroxy-19-nor-4-androsten-3-one
Oestradiol	=	3 β ,17 β -dihydroxy-19-nor-1,3,5(10)-androstatriene
Progesterone (Pr)	=	4-pregnene-3,20-dione
Pregnenolone Acetate	=	3 β -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

$R_1 = R_2 = R_4 = \text{OH}, R_3 = \text{H}$ 19-hydroxycortexolone

$R_1 = R_2 = R_3 = \text{OH}, R_4 = \text{H}$ 11 β -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 stereochemistry

ABBREVIATIONSAbbreviations of Units

amu	atomic mass unit
°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 _{11β}	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 ₀	Feed-medium glucose concentration (g/l)
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
tlc	Thin Layer Chromatography
UV	Ultra-violet
μ	Specific Growth Rate (h ⁻¹)