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TOWARDS MOLECULAR BIOLOGICAL CHARACTERISATION OF THE GENES FOR STEROL AND BILE ACID METABOLISM IN NOCARDIOFORM BACTERIA

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A DISSERTATION TO THE FACULTY OF SCIENCE, UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECNOLOGY

JOHANNESBURG 1989

ABSTRACT

The steroids are a diversified class of oxygenated tetracyclic isoprenoid derivatives characterized by a cyclopentanoperhydrophenonthrene ring system. Two important groups of steroids are the sterols, of which cholesterol is the most important, and bile acids, which are converted from cholesterol.

Commercial biotechnology operations dealing with steroids, centre around the use of microorganisms for transformations of steroid substrates into useful intermediates and final products. One group of bacteria capable of the before mentioned are the nocardiform bacteria. Cloning the genes of these organisms for the interconvertion of steroids and bile acids to pharmacologically important intermediates by complementation of the appropriate mutation was the main interest of this work. Mutagenesis using U.V.; NTG and EMS as mutagens, and the modified penicillin selection technique, resulted in several mutants, notably KD 1, which had lost its ability to utilize several carbon sources. The identification and determination of the suitability of these carbon sources was performed. Conditions of transformation of this mutant were optimized.

The availability of a suitable vector led to the partial construction of a library of nocardioform chromosomal DNA in *E.coli* to clone the gene/genes of interest.

DECLARATION

I declare that this dissertation is my own, unaided work.

It is being submitted for the degree of Master of Science in Biotechnology in the University of the Witwatersrand, Johannesburg.

It has not been submitted for any degree or examination in any other University.

Katrina Jo Downing

K Downing

28th day of February, 1989

TO MY FAMILY AND RODERICK WESTWOOD

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1.0 INTRODUCTION

The steroids belong to a class of lipid compounds called terpenoids (and other names such as terpenes, polyisoprenoids or isopentenoids). They are a diversified class believed to be derived from isoprene, C_5H_8, and are characterized by the cyclopentanoperhydrophenanthrene ring system of Figure 1.

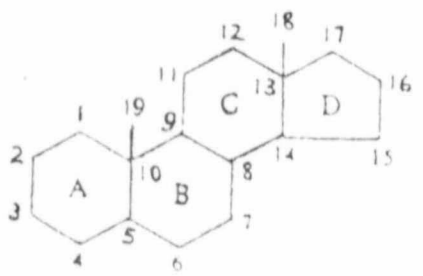


Figure 1. The steroid ring system. Smith 1984

The sterols, a subgroup of steroids, are a class of crystalline alcohols containing between 27 and 30 carbon atoms. They all possess a 3β- hydroxy group and an endocyclic double bond, usually in the 5,6 position, together

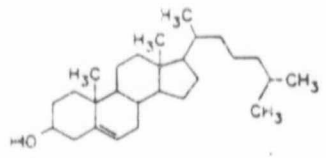
with a side chain which exhibits various degrees of branching and unsaturation. (Templeton 1969).

The sterols are widely distributed in nature and are present in practically all living organisms including bacteria. Cholesterol is the most important and widespread of the sterols. It is the precursor of the bile acids and the sex and adrenocortical hormones. The most abundant plant sterol is stigmasterol, first isolated from the Calabar bean but more plentifully available from soybean oil. Sitosterol is also a sterol. (Templeton 1969). Other sterols include campesterol and ergosterol.

See Figure 2 for structures of some sterols.

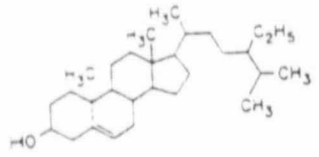
Cholesterol

$C_{27}H_{46}O$
M = 386.67 g/mol
CARN [57-88-5]



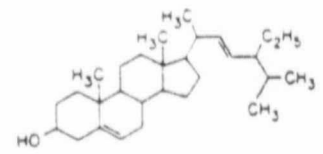
β-Sitosterol

$C_{29}H_{50}O$
M = 414.72 g/mol
CARN [83-46-5]



Stigmasterol

$C_{29}H_{48}O$
M = 412.70 g/mol
CARN [83-48-7]



Sodium Taurocholate

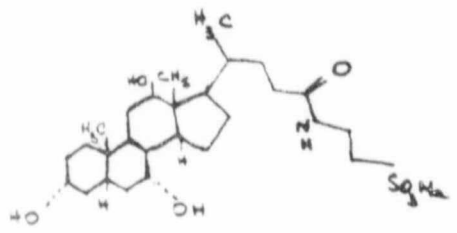


Figure 2. Structures of some sterols

Bile acids are C-24 to C-28 carboxylic acids with a steroid nucleus containing hydroxylic substituents and part or all of the side chain of 5β-cholestane. (Whiting 1986). Bile acids contain hydroxyl groups, which are substituted at position C-3, C-7, C-12 of the steroid nucleus. The three major bile acids are cholic acid, chenodeoxycholic acid and deoxycholic acid. Usually bile acids are enzymatically conjugated with either of the amino acids glycine or taurine in human bile. There are thus six major bile acids in man, namely the glycine and taurine conjugates of cholic acid, chenodeoxycholic acid and deoxycholic acid. The bile acid sodium taurocholate was used in the work for this thesis.

Steroids are vital in many ways to life of eukaryotic organisms. The sterols are precursors of other steroids and are essential for membrane stability and cell growth. The bile salts are essential for lipid digestion and absorption. Additionally there are two classes of hormones that are steroidal in nature, namely the sex hormones and the adrenocortical hormones. It is the pharmacological interest in these hormones which prevails in current applications of biotechnology to the steroids. (Smith 1984).

The sex hormones fall into three chemically and physiologically distinct classes. The estrogens and progestogens regulate various functions of the female reproductive system and the androgens are male hormones.

The adrenocortical hormones are produced by the cortex of the adrenal gland. They are vital in the metabolism of water, proteins and carbohydrates. There are seven corticoid hormones of which cortisone has important medicinal applications.

Microbial transformation of specific steroid substrates is the centre of commercial biotechnology operations dealing with steroids and has resulted in major contributions to technology, medicine and science. There are currently two major biotechnological applications dealing with the steroids. These applications involve the use of microorganisms for processing raw materials into useful intermediates for general steroid production and that of specific transformations of steroid intermediates to finished products. (Smith 1984).

The first successful application of biotechnology to the preparation of useful steroids had to do with the synthesis of the adrenocortical hormones and their more powerful and therapeutically selective synthetic analogs. (Charney and Herzog 1967). Cortisone has a powerful antiinflammatory activity which was discovered in 1949. Synthesis of cortisone did not include microorganisms initially. However, Murray, Peterson, Perlman and Fried laid the basis for the application of microbiology to the synthesis of antiinflammatory steroids. (Charney and Herzog 1967).

The world market for finished steroids appears to be increasing both in amount of production and in monetary value. Sales of antiinflammatory steroids in the USA of $120 million in 1959 increased to $215 million by 1968. Markets for other steroids including male and female sex hormones, anabolic agents, anesthetics, antialdosterone agents, anticancer agents and antiandrogenic agents also continue to grow. (Smith 1984). Three general processes are used to produce finished steroid products.

1. Direct isolation from natural sources such as the recovery of conjugated estrogens from horse urine and of cardiotonic steroids from the plants *Digitalis*.

2. Partial synthesis from steroid raw materials of animal and plant origins. The partial synthesis of steroid hormones and their analogs is the most important process with respect to microbial biotechnology.
3. Total synthesis from non steroidal materials.

All three of these processes are commercially operated but it is only the second two that use microbial transformations.(Kieslich 1980, Smith 1984)

There is a diversity of enzymic reactions accomplished by microorganisms on steroids.
The most important category of microbiological transformations is oxidation. There are four oxidation reactions of commercial importance, hydroxylation, alcohol oxidation, 1 - dehydrogenation and carbon - carbon bond scission.
Microbial hydroxylations at almost every possible position in the steroid nucleus are known including 10β - hydoxylation of 19 - norsteroids and 14β- hydroxylation of synthetic 14β - steroids. (Smith 1984). There are three microbial hydroxylations of commercial importance, 11α - ,11β - and 16α - hydroxylations.
Microbial scissions of carbon - carbon bonds are very important, as cleavage of sterol side chains to useful C_{19} intermediates and of progesterone side chain to testololactone derivatives are commercial processes.
Microbial reductions of ketones are also of commercial interest whereas other reactions such as isomerizations and conjugations are of little commercial importance.

Cholesterol and deoxycholic acid from slaughter house animals were used originally as starting material for microbial transformations. For reasons of cost additional raw materials are used. These include Solasodine and Tomatidine derived from *Solanum* species, Smilagenin from *Agave* species and Hecogenin from sisal plants. The phytosterols diosgenin from Mexican and other *Dioscorea* plants and stigmasterol from soybeans give useful C_{21} intermediates from the finished C_{18}- C_{19}- and C_{21} - steroids are manufactured today.

Cholesterol, from wool grease and soybean sitosterol are used for microbial degradations of the sterol side chains yielding useful C_{19} steroid intermediates. Microbial degradations of sitosterol and of cholesterol yield C_{19} steroids used for production of other C_{19} steroid sex hormones and anabolic agents. (Kieslich 1980, Smith 1984).

It has been known since 1913, that numerous microorganisms can utilize sterols such as cholesterol and β-sitosterol as sole source of carbon. However, they have a serious drawback as they do not only degrade the side chain, by the mechanism of β oxidation, but also cleave the steroid skeleton by 9 α hydroxylation, which is undesirable and of no commercial value. (Kieslich 1980). In 1965, Sih and workers elucidated the degradation method and described a method for avoiding the undesirable reactions described above. A suitable cholesterol-like substrate such as 19-hydroxy-cholesterol was used as a substrate and the side chain was degraded to a 17 ketosteroid to yield estrone. (Charney and Herzog 1967, Kieslich 1980).

Three different methods have been developed to selectively cleave the side chain of sterols by microorganisms. These processes are based on the

inhibition of the key enzymes C-1(2)-dehydrogenase and 9 α hydoxylase involved in steroid ring degradation.

The methods employed to inhibit one or more of these enzymes are the following:

1. Structural modification of the substrate as described by Sih and workers (cited in Charney and Herzog 1967) preventing enzymic attack on the ring system. Some sterols with chemically modified structures are 19-hydroxysterols, 19 - norsterols and 4,hydroxycholestenone.

2. The use of chemical enzyme inhibitors.

Numerous processes for the selective side chain cleavage of sterols employing enzyme inhibitors have been developed. Chemicals used as enzyme inhibitors included lipophilic chelating agents such as αα' Dipyridyl (Nakamatsu *et al* 1980), autoxidizable redox dyes, inorganic SH reagents and metal ions.

3. Mutation of the microorganisms

Microorganisms have been produced by mutagenesis that are capable of selectively degrading the sterol side chain. These mutants are biochemically blocked from degrading the nucleus and can be used to efficiently produce steroids from sterols without the necessity of modifying the substrate or of adding chemical inhibitors. These mutants have been found to have potential industrial use, efficiently converting sterols to products some of which are useful as intermediates in the manufacture of medically important steroids. Several researchers have reported the degradation of sterols by mutants to industrially important intermediates. Wovcha *et al* 1978, Hill *et al* 1982, Nakamatsu *et al* 1983, and Ferreira *et al* 1984 are just a few of the researchers using this process of selective side chain degradation.

The sterols play a vital role in oral contraceptives of which two are generally available namely the combined pill and the gestagen pill. These products consist of an estroger and/or a gestagen which is a synthetic substance having biological effects resembling progesterone. Two forms of gestagens are available: those derived from testosterone by replacing the methyl group of the carbon 19 atom with a hydrogen atom (19-nortestosterone) and those derived from 17 - acetoxy progesterone. The 19-nortestosterones in use today are norethisterone, lynestrenol, ethynodiol diacetate and d-norgestrel. Only one 17-acetoxyprogesterone is currently available for contraception and this is medroxy-progesterone acetate. (Llewellyn - Jones 1978)

Cholesterol decomposing microorganisms are distributed in a wide range of genera of actinomycetes, other bacteria, molds and yeasts. Some of the genera include *Pseudomonas*, *Corynebacteria*, *Arthrobacter*, *Streptomyces*, *Mycobacterium*, *Nocardia* and *Rhodococcus*. A number of cholesterol decomposing strains belong to the genera *Mycobacterium*, *Nocardia* and *Rhodococcus* known as the nocardioform bacteria whereas in the other genera, the decomposing strains appear incidentally.(Arima *et al* 1969).

In previous years the taxonomy of the nocardioform bacteria was extremely poor. In the seventh edition of Bergey's manual, the nocardioform bacteria were classified in the genera *Nocardia* and *Actinomycetes* belonging to the family Actinomycetaceae. The genus *Nocardia* was poorly defined and contained bacteria that had little in common. In the late seventies efforts were directed towards establishing a better classification of the actinomycetes. They have been classified into 10 families and 30 genera, based primarily on morphological, chemical and spore characteristics.

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