any P-450 used in this manner will depend on the factors outlined here, substrate specificity and electron transfer efficiency of the redox donor(s). Both of these factors can be subject to further bioengineering.

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Microbial transformations of steroids
Kelvin E. Smith, Farjad Ahmed and Tony Antoniou
Department of Biochemistry, Queen Mary and Westfield College, London E1 4NS, U.K.

Introduction
Steroid research in the U.S.A. and in the U.K. was stimulated greatly in the early years of the Second World War by rumours, from the Polish underground, that the Luftwaffe was administering corticosteroids to its pilots. Increased resistance to shock, enhanced night vision and greater tolerance of high altitude were attributed to the treatment, although the claims were never substantiated. The real physiological impact of this research had to wait until 1949, when Hench, Kendall and associates at the Mayo Clinic in the U.S.A. announced that cortisone (Figure 1; 1) has anti-inflammatory properties that are useful in treating degenerative joint diseases, such as rheumatoid arthritis [13]. Oxygen functionality at 11β was found to be mandatory for maximum activity. These observations stimulated a fresh burst of interest in the synthesis of corticosteroids and a search for potent analogues.

Chemical synthesis of cortisone
In the 1940s bile acids were the only practical source of starting material for the synthesis of hormonal steroids. Chemical transformation of deoxycholic acid to corticosteroids requires the transposition of the 12α-hydroxyl to the 11-posi-

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route was never commercialized, being superseded by a similar route starting from hecogenin, which possesses a 12-keto group.

Microbial approach to the synthesis of corticosteroids

The eventual solution to the 11-hydroxylation problem came from a most unusual and highly unexpected quarter. In 1952, Peterson and Murray, at Upjohn, found that aerated cultures of the Mucorales fungi *Rhizopus arrhizus* and *R. nigricans* were capable of direct 11α-hydroxylation of progesterone (4) [5, 6]. Virtually simultaneously, Fried and co-workers at the Squibb Institute reported the same reaction with *Aspergillus niger* on progesterone, deoxycorticosterone, 11-deoxy-17α-hydroxycorticosterone (Reichstein's substance S) and 17α-hydroxyprogesterone [7]. The inversion of the hydroxyl from the 11α to the 11β configuration is readily achieved by simple oxidation-reduction chemistry. A few years later (1955), direct microbial steroid 11β-hydroxylation was reported by Schull and Kita at Pfizer who found that *Curvularia lunata* transformed efficiently a virtually identical spectrum of progesterone substrates to *A. niger* [8]. The replacement of chemical 11-hydroxylation by direct microbial transformation resulted in much improved product yields, which in turn lowered significantly the cost of production of hydrocortisone; for example from $200 per g in 1948 (Merck bile acid route) to $35 per g in 1951 (Glaxo hecogenin route) to only $3.50 per g in 1955 (microbial 11-hydroxylation). Not surprisingly, clinical use of these compounds increased greatly during that period.

Site-selective hydroxylation of steroids by micro-organisms

Microbiological regio- and stereoselective hydroxylation of every steroid skeletal position is now possible [9]. Thus, 9α- and 16α-hydroxylation, which are key steps in the industrial synthesis of hydrocortisone analogues, are now accomplished exclusively microbially. A patent has been taken for the preparation of novel anticholesterolaeic compounds from β-sitosterol, which involves a 9α-hydroxylation step with a strain of *Mycobacterium* to produce 9α-hydroxy-3-oxo-stigmaster-4,24(25)-dien-26-oic acid [10]. 7α- Hydroxyandrosterone is important in the manufacture of diuretics. Its microbial production from 3α,7α-dihydroxy-5β-cholanic acid or its salts has been patented [11].

Many fungi are capable of steroid 14α-hydroxylation [9, 12]. It is a distinct possibility that
these hydroxylation agents could be used industrially in the synthesis of 14α-hydroxysterols, which show promising anticancer properties.

**Fungal steroid hydroxylases are site-selective cytochrome P-450 monooxygenases**

The fungal xenobiotic steroid hydroxylases are site-selective cytochromes P-450 [13-18] that are distinct from lanosterol 14α-demethylase cytochrome P-450, an enzyme that catalyses the oxidative demethylation of lanosterol to 4,4-dimethylcholesta-8,24-diene-3-one in the biosynthesis of the fungal membrane sterol, ergosterol.

The involvement in filamentous fungi of cytochrome P-450 in exogenous steroid hydroxylation was unknown until 1977 when the inducible microsomal, membrane-bound, progesterone 11α-hydroxylase system of *R. nodulans* was first described [13]. The cytochrome P-450-containing electron transport system has been resolved into three components and is similar in composition to the bacterial and mitochondrial systems. It consists of cytochrome P-450 (46 kDa) as the terminal oxygenase and an iron-sulphur ferredoxin (rhizoporedoxin) and an FAD-flavoprotein, ferridoxin reductase (NADPH-rhizoporedoxin reductase), as the electron carriers [19, 20]. Progesterone hydroxylation was not demonstrated in a reconstituted system.

Microsome fractions, capable of in vitro steroid hydroxylation, have been prepared from various filamentous fungi including *A. ochraceus* (11α), *Botryosphaeria obtusa* (7β), *Cochliobolus lunatus* (11β), *C. lunata* (11β), *Mucor piriformis* (14α) and *Phycomyces blakesleeanus* (7α). Hydroxylase site-selectivity was retained by the individual cytochromes P-450. The hydroxylation systems have not been resolved and the cytochromes P-450 have not been purified. The true physiological substrates of these enzymes are not known.

**Progesterone-hydroxylase cytochromes P-450 of *A. fumigatus***

Much research has been done on the sterol 14α-demethylase cytochrome P-450 of *A. fumigatus* and its inhibition by azole fungicides. Data point to cytochrome P-450 heterogeneity in this organism. Exogenous progesterone is hydroxylated efficiently producing, after 3 h of incubation, 11α- and 15β-hydroxyprogesterone as major products and 7β- hydroxyprogesterone, 7β,15β- and 11α,15β-dihydroxyprogesterone as minor products. After 72 h, dihydroxylation is complete, and 7β,15β- and 11α,15β-dihydroxyprogesterone are the only metabolites present.

* *A. fumigatus* microsomes, prepared by careful homogenization with abrasives [21], and then by Ca²⁺ precipitation of the post-mitochondrial supernatant [22], exhibit a typical reduced cytochrome P-450 CO difference absorbance spectrum with a maximum at 446 nm and a minimum at 490 nm, and showing no contamination by cytochrome oxidase (a common problem in fungal cytochrome P-450 preparations). These preparations also give the typical type I progesterone substrate-binding spectrum exhibited by benzo[a]pyrene substrate binding to yeast cytochrome P-450 [23].

When incubated at 22°C in the presence of NaIO₄, these microsome preparations catalyse the efficient hydroxylation of progesterone producing only 7β-, 11α- and 15β-hydroxyprogesterone. Boiling, bubbling with carbon monoxide and incubation with the azole fungicide ketoconazole totally inhibits hydroxylation, as expected of a cytochrome P-450-catalysed system. Optima for NaIO₄ and for progesterone are 1 mM and 2 mM respectively.

The progesterone hydroxylases are constitutive and non-amplifiable. Microsomes from 4-day-old progesterone preincubated mycelia (10 μgml⁻¹ for 16 h) transform progesterone identically to non-preincubated controls.

The presence of multiple forms of progesterone hydroxylase cytochrome P-450 in *A. fumigatus* is revealed by ketoconazole inhibition data. Progesterone 7β-hydroxylation is more sensitive to the inhibitor than 11α- or 15β-hydroxylation. Ketoconazole IC₅₀ values for steroid hydroxylase inhibition are between one and two orders of magnitude higher than for lanosterol demethylase inhibition. The progesterone hydroxylases turn over rapidly in the membrane. Microsomes prepared from mycelia preincubated for 18 h with cycloheximide (5 μgml⁻¹), and progesterone (10 μgml⁻¹) transform poorly compared with non-preincubated controls.

The NaIO₄ requirement for progesterone hydroxylation can be substituted by other peroxo-compounds and by NADPH and O₂, but not by NADH. Identical sets of metabolites are produced in all incubations.

**Purification of *A. fumigatus* progesterone hydroxylase cytochromes P-450**

* A. fumigatus progesterone hydroxylase cytochromes P-450 are solubilized by 1% Triton N-101 for 6 h at
0°C and precipitated by 70% (w/v) (NH₄)₂SO₄. These preparations hydroxylate progesterone with NaIO₄ and with NADPH. The NADPH-dependent hydroxylation is stimulated by cloned human placental cytochrome P-450 reductase and by cloned beef liver cytochrome b₅. A high degree of purification is obtained by batch-column chromatography on DEAE-cellulose (0.25–0.5 M KCl) followed by Red-3 affinity columns (0.1–0.5 M KCl). The major protein bands seen on SDS/polyacrylamide gels have molecular masses of around 55–58 kDa.

**Future prospects and applications**

Regio- and stereoselective transformation of structurally complex substrates and the creation of chirality are major objectives in modern synthetic organic chemistry. Accomplishment of these objectives usually demands sophisticated and time-consuming chemistry. Many steroid-transforming fungi also site-selectively hydroxylate a range of non-steroid substrates, providing the hydrophobicity is high. Not surprisingly, these organisms are finding their way into general pharmaceutical production in a bid to reduce costs and to produce chiral products. Soon they will be used in the agrochemical industry to produce new herbicides and pesticides, and in the food industry to produce new flavours and essences.

Active cytochromes P-450 can be synthesized in large quantities in *Escherichia coli* and yeasts using modern cloning technology. The stability and efficiency of these proteins can also be improved by this technology. Once cloned fungal steroid hydroxylase cytochromes P-450 become readily available, these enzymes could be immobilized and used in bioreactors. The requirements for NADPH and molecular oxygen and for cytochrome P-450 reductase, which currently prevent the use of immobilized cytochromes P-450, could be replaced by cheap and plentiful peroxo-compounds.

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