Total biosynthesis of hydrocortisone from a simple carbon source in yeast

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We report on the production of hydrocortisone, the major adrenal glucocorticoid of mammals and an important intermediate of steroidal drug synthesis, from a simple carbon source by recombinant *Saccharomyces cerevisiae* strains. An artificial and fully self-sufficient biosynthetic pathway involving 13 engineered genes was assembled and expressed in a single yeast strain. Endogenous sterol biosynthesis was rerouted to produce compatible sterols to serve as substrates for the heterologous part of the pathway. Biosynthesis involves eight mammalian proteins (mature forms of CYP11A1, adrenodoxin (ADX), and adrenodoxin reductase (ADR); mitochondrial forms of ADX and CYP11B1; 3β-HSD, CYP17A1, and CYP21A1). Optimization involved modulating the two mitochondrial systems and disrupting of unwanted side reactions associated with *ATF2, GCY1,* and *YPR1* gene products. Hydrocortisone was the major steroid produced. This work demonstrates the feasibility of transfering a complex biosynthetic pathway from higher eukaryotes into microorganisms.

Hydrocortisone (11β,17α,21-trihydroxy-4-pregnene-3,20-dione) is the major steroid in mammals. It has a weak glucocorticoid effect and is the preferred starting material for synthesis of drugs with potent anti-inflammatory, abortive, or antiproliferative effects. Total chemical synthesis of hydrocortisone, initially reported in 1952 by R.B. Woodward and co-workers, involves ~40 steps[1]. Currently, hemi-synthesis starting from naturally occurring sterols is used for industrial manufacture of this drug. It involves a rather sophisticated multi-step chemical procedure and a microbial bioconversion step. Thus, there is a need for innovative synthetic routes.

Here we report the first total biosynthesis of hydrocortisone by a recombinant microorganism. It involves natural yeast biosynthetic pathways, a yeast pathway rerouted using one plant enzyme, and five additional enzymatic steps catalyzed by eight mammalian proteins. This mammalian portion of the pathway mimics the adrenal biosynthesis of this hormone and includes mainly membrane-bound enzymes: four members of the P450 superfamily of monooxygenases, 3β-hydroxy steroid dehydrogenase/isomerase (3β-HSD), and three electron carriers[2].

In a previous study, we described the biosynthesis of progesterone in engineered *S. cerevisiae* producing plant Δ7-reductase (DWF5), mature forms of CYP11A1 (P450 side chain cleaving), ADX (FDX1), ADR (FDXR), and 3β-HSD[3]. Results presented here demonstrate the successful reconstruction of the entire pathway allowing hydrocortisone production from a simple carbon source. Major hurdles solved in the course of this work include (1) self-production of a suitable substrate for CYP11A1, (2) targeting of mitochondrial P450s and associated carriers, (3) metabolic flux equilibration of the artificial biosynthetic pathway, (4) identification and circumvention of host-related side reactions diverting intermediates into metabolic dead ends, and (5) toxicity of certain biosynthetic intermediates for yeast. Although other examples of pathway engineering exist in the literature[4,5], we describe here the engineering of the up to now most complex biosynthetic pathway into a eukaryotic microorganism and the first instance that also includes several coupled membrane enzymes.

Results

Experimental strategy to generate strains capable of producing hydrocortisone. As illustrated in Figure 1 and described in the Experimental Protocol, the mammalian-specific part of the hydrocortisone biosynthetic pathway was introduced through both plasmid and genomic integration-based genetic engineering techniques into strains where ergosterol biosynthesis had previously been rerouted to produce suitable CYP11A1 substrates[3]. Side reactions leading to steroid biosynthesis dead ends (galactose-inducible crystalline-like yeast protein, Gcy1p; aldo-keto reductase, Ypr1p; and alcohol *O*-acetyltransferase, Atf2p-dependent) were also identified and inactivated as required. Finally, expression levels were adjusted

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Table 1. List of yeast strains assembled in this study and relevant genotypes

Strain[a]	Relevant feature[b]
FY1679-18b	*MATa, ρ⁺, GAL, ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1*
FY1679-28c	*MATα, ρ⁺, GAL, ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1*
TGY170	FY1679-28c *gcy1*::*LEU2*
TGY197	TGY170 *ypr1*::*URA3*
TGY195#4	FY1679-18b *ypr1*::*URA3*
TGY212-1	TGY195#4 *ypr1*::P*TEF1*-CYP21A1-T*PGK1*
TGY243-1	TGY212-1 *gcy1*::*URA3*
TGY245-2D	TGY243-1 *gcy1*::P*TDH3*-CYP21A1-T*PGK1*
TGY260-A	TGY245-2D *LEU2*::P*CYC1-ARH1*-T*PGK1*
CDR06[c]	*MATα; ρ⁺, GAL, ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1; ade2*::P*GAL10/CYC1*-Δ7 reductase-T*PGK1*
CDR07[c]	*MATα; ρ⁺, GAL, ura3-52, trp1-Δ63, his3-Δ200, leu2-Δ1*,erg5::P*PGK1-HYGRO*^{R}*; ade2:PGAL10/CYC1*-Δ7 reductase-T*PGK1*
YCC4[d]	*MATα, ura3-52, trp1-Δ63, his3-Δ200, LEU2*::P*CYC1-ARH1*-T*PGK1 ypr1*::P*TEF1*-CYP21A1-T*PGK1, gcy1*::P*TDH3*-CYP21A1-T*PGK1, ade2*::P*GAL10/CYC1*-Δ7 reductase-T*PGK1*
YCC5[c]	*MATα, ura3-52, trp1-Δ63, his3-Δ200, LEU2*::P*CYC1-ARH1*-T*PGK1, gcy1*::P*TDH3*-CYP21A1-T*PGK1, ade2*::P*GAL10/CYC1*-Δ7 reductase-T*PGK1*
UCYA	CDR06 *TRP1*::P*TEF1*-CYP17A1-T*PGK1*, *HIS3*::P*TDH3-COX6*$_{pre}$-matADX-T*PGK1*
UCYB	YCC5 *HIS3*::P*TDH3-COX6*$_{pre}$-matADX-T*PGK1*, *HIS3*::P*TDH3-COX6*$_{pre}$-matADX-T*PGK1*
UCYC	YCC4 *HIS3*::P*TDH3-COX6*$_{pre}$-matADX-T*PGK1*, *HIS3*::P*TDH3-COX6*$_{pre}$-matADX-T*PGK1*
UCYD[3]	*MATα, ura3-52, trp1-Δ63, his3-Δ200, LEU2*::P*CYC1-ARH1*-T*PGK1 ypr1*::P*TEF1*-CYP21A1-T*PGK1, gcy1*::P*TDH3*-CYP21A1-T*PGK1, ade2*::P*GAL10/CYC1*-Δ7 reductase-T*PGK1*
UCYE	UCYD *LEU2*::P*CYC1-ARH1*-T*PGK1*
UCYF	UCYD *LEU2*::P*TEF1-ARH1*-T*PGK1*
UCYG	UCYC *atf2*::P*TEF1*-G418^R
UCYH	UCYB *atf2*::P*TEF1*-G418^R
UCYI	UCYE *atf2*::P*TEF1*-G418^R
UCYJ	UCYF *atf2*::P*TEF1*-G418^R

[a]FY1679-28c and FY1679-18b are described in ref. 22. UCY, Universal Corticoids Yeast.
[b]Functional locus or genes are shown in italic uppercase letters; nonfunctional ones are in italic lowercase letters. Promoters and terminators are represented as follows: P*TDH3*, *TDH3* promoter; T*PGK1*, *PGK1* terminator, etc. Insertions are indicated by a double colon (::).
[c]Spore clone of a cross CA10 × FY1679-18b (this study).
[d]Spore clone of SB14 (= TGY260-A × CDR07) (this study).
[e]Spore clone of YSA2 (= TGY245-2D × UCYC) (this study).

to optimize steroid channeling to hydrocortisone. Genetic structures were assessed by molecular biology techniques (PCR, Southern blotting, sequencing). Expression and activity of proteins were verified by bioconversion (cytochrome P450 enzymes), by sterol profiling (Δ7-reductase), or by western blots of mature form ADX (mat-ADX) and ADR, *COX6* $_{pre}$ mat-ADX[6] (where *COX6* $_{pre}$ is the presequence of cytochrome *c* oxidase subunit VI), and ADR-related homolog (Arh1p)[7].

Assembly of initial strains carrying genetic material encoding the whole pathway and producing hydrocortisone. Strains UCYA, B, C, and D producing CYP11A1 substrates and possessing different combinations for *GCY1* and *YPR1* disruptions (see next section for details) were co-transformed with two plasmids, multicopy and monocopy, respectively. Accordingly, UCYA pCV29/pCC22, UCYB pCV29/pCC12, UCYC pCV29/pCC12, and UCYD pCV29/pCC12 carried all the genetic information necessary for self-sufficient hydrocortisone synthesis (Fig. 1, Tables 1 and 4). All strains were capable of producing hydrocortisone as well as other steroids at ~1 μg/ml (Fig. 2 and Table 2). Interestingly, only hydrocortisone, 11-deoxycortisol (the last intermediate), corticosterone (11β,21-dihydroxyprogesterone, obtained by CYP21A1 (21-steroid hydroxylase) acting on

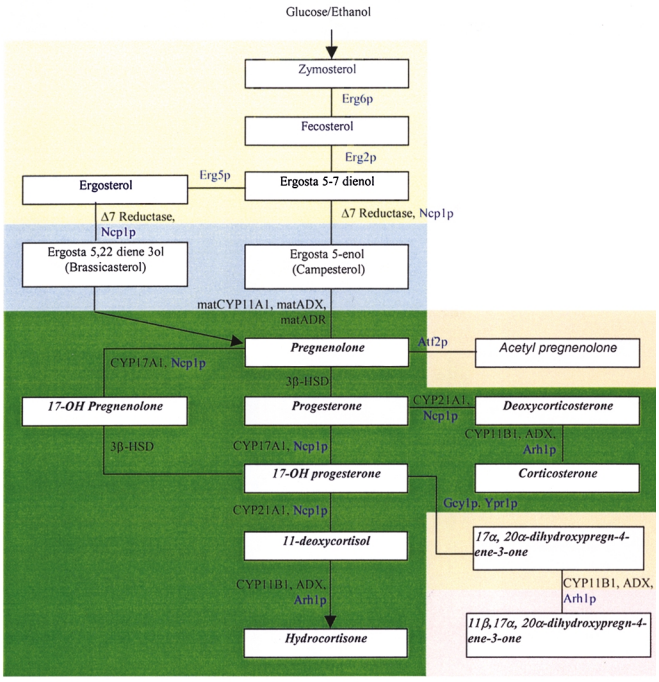

Figure 1. Reconstitution of the hydrocortisone biosynthetic pathway in *S. cerevisiae*. Connection of the ergosterol pathway with the hydrocortisone pathway. Ncp1p, NADPH P450 reductase; ADX, adrenodoxin; ADR, adrenodoxin reductase; Arh1p, adrenodoxin reductase-related homolog; Atf2p, alcohol *O*-acetyltransferase (acetylpregnenolone acetyltransferase); CYP11A1, P450 side chain-cleaving; CYP17A1, 17α-steroid hydroxylase; CYP21A1, 21-steroid hydroxylase; CYP11B1, 11β-steroid hydroxylase; Erg2p, sterol C8-C7 isomerase; Erg6p, *S*-adenosyl methionine Δ-24-sterol-C-methyltransferase; Erg5p, Δ22(23)-sterol desaturase; 3*β*-HSD, 3*β*-hydroxy steroid dehydrogenase; Gcy1p and Ypr1p, aldo-keto reductases. Yeast sterols (yellow), steroids (green), yeast-made derivatives from steroids (orange), Δ7-reductase products (blue), side-product made by steroidogenic enzymes (purple); yeast endogenous proteins are marked as the respective gene products.

Table 2. Steroid production of UCY strains

Strains and relevant genotypes[a]	Steroids (μg/ml) Hydrocortisone	Corticosterone	11-Deoxycortisol	17α,20α-Dihydroxypregn-4-ene-3-one	Pregnenolone acetate	Pregnenolone	Progesterone
UCYA (*ATF2*, *GCY1*, *YPR1*)	0.97 ± 0.08	1.19 ± 0.04	0.15 ± 0.02	<0.1	0.40	<0.05	<0.1
UCYB (*ATF2*, gcy1, *YPR1*, *ARH1*$^{+}$)	1.25 ± 0.23	1.35 ± 0.19	<0.1	<0.1	0.52	<0.05	<0.1
UCYC (*ATF2*, *GCY1*, *ypr1*, *ARH1*$^{+}$)	0.67 ± 0.03	0.76 ± 0.03	<0.1	<0.1	0.42	<0.05	<0.1
UCYD (*ATF2*, *gcy1*, *ypr1*)	0.66 ± 0.02	1.04 ± 0.08	0.24 ± 0.02	<0.1	0.58	<0.05	<0.1
UCYE (*ATF2*, *gcy1*, *ypr1*, *ARH1*$^{+}$)	0.5 ± 0.1	0.7 ± 0.1	<0.1	<0.1	0.45	<0.05	<0.1
UCYF (*ATF2*, *gcy1*, *ypr1*, *ARH1*$^{++}$)	1.5 ± 0.1	0.75 ± 0.04	<0.1	<0.1	0.26	<0.05	<0.1
UCYG (*atf2*, *GCY1*, *ypr1*, *ARH1*$^{+}$)	4.00 ± 0.01	1.33 ± 0.01	0.55 ± 0.01	1.8 ± 0.1	<0.05	traces	traces
UCYH (*atf2*, *gcy1*, *YPR1*, *ARH1*$^{+}$)	9.49 ± 0.04	3.14 ± 0.14	1.07 ± 0.15	<0.1	<0.05	traces	traces
UCYI (*atf2*, *gcy1*, *ypr1*, *ARH1*$^{+}$)	11.5 ± 0.3	4.0 ± 0.1	1.10 ± 0.02	<0.1	<0.05	traces	traces
UCYJ (*atf2*, *gcy1*, *ypr1*, *ARH1*$^{++}$)	9.6 ± 0.5	3.2 ± 0.2	0.8 ± 0.3	<0.1	<0.05	traces	traces

[a]Strains are co-transformed with plasmids pCV29 and pCC12, except UCYA, for which pCC22 replaced pCC12. Deoxycorticosterone (21-hydroxyprogesterone) and 17α-hydroxyprogesterone are not detected in any of the UCY strains. A steroid that is probably the product of 21- or 11-hydroxylation of 17α,20α-dihydroxypregn-4-ene-3-one is also detected in UCYG strain only. *ATF2*, *GCY1*, *YPR1*: Functional copies of these genes are present. *atf2*, *gcy1*, *ypr1*: The corresponding genes are non-functional. *ARH1*$^{+}$: A second copy of *ARH1* gene is present under the control of the *CYC1* promoter. *ARH1*$^{++}$: A second copy of *ARH1* gene is present under the control of the *TEF1* promoter (a stronger promoter). The different strains reach a final A_{600nm} close to 50 (15 g/L dry cell weight) after 172 h (Experimental Protocol). Except for pregnenolone acetate, data are in triplicate.

progesterone), and acetyl pregnenolone (produced by Atf2p activity at 0.4–0.6 μg/ml) could be detected, indicating that the flux toward hydrocortisone was fairly balanced. Minimizing 11-deoxycortisol accumulation by increasing CYP11B1 (11β-steroid hydroxylase) activity and eliminating pregnenolone acetylation by disruption of the *ATF2* gene[8] resulted in further improvements (see below).

Purpose of *GCY1*- and *YPR1*-dependent side reactions. It has been shown that as a result of a side reaction competing with further conversion of 17α-hydroxyprogesterone by CYP21A1, incubation of 17α-hydroxyprogesterone with wild-type or recombinant yeast expressing CYP17A1 (17α-steroid hydroxylase) yields a product identified as 17α,20α-dihydroxypregn-4-ene-3-one[9,10]. A similar activity has been reported in mammalian endocrine tissues[11]. Analysis of the yeast genome led us to identify Gcy1p and Ypr1p as candidate enzymes for this 20α-hydroxysteroid dehydrogenase activity (20α-HSD) because of their high identity with the bovine protein (44 and 43%, respectively). Two yeast strains with *GCY1* (TGY170) or *GCY1* and *YPR1* (TGY197) deletions were constructed. Keto-reduction was evaluated in extracts from both strains using methylglyoxal, glyceraldehydes, or 17α-hydroxyprogesterone as substrate. Activities measured with methylglyoxal or glyceraldehydes were reduced to variable degrees in extracts lacking *YPR1* and/or *GCY1* gene products, regardless of the carbon source used for growth (Table 3A). In contrast, disruption of both *GCY1* and *YPR1* resulted in no traceable formation of 17α,20α-dihydroxypregn-4-ene-3-one from 17α-hydroxyprogesterone, whether yeast cells were grown on galactose (a known *GCY1* inducer[12]) or glucose (Table 3A).

Disrupted strains TGY245-2D (*gcy1-Δ*, *ypr1-Δ*) and TGY212 (*ypr1-Δ*) expressing CYP21A1 were compared to the corresponding control strain (FY1679-28c/pTG10497). Both converted 17α-hydroxyprogesterone into 11-deoxycortisol with different yields (Table 3B), TGY245-2D being the most efficient. No 17α,20α-dihydroxypregn-4-ene-3-one was detected, even after prolonged incubation. In addition, the bioconversion yield into 11-deoxycortisol was substantially improved from 18.7% to 71.7% of the substrate with respect to 21-hydroxylase activity. Thus, strain TGY245-2D and its derivative TGY260-A, which overexpresses Arh1p, were used as background strains for reconstitution of the entire pathway (see earlier and Table 1).

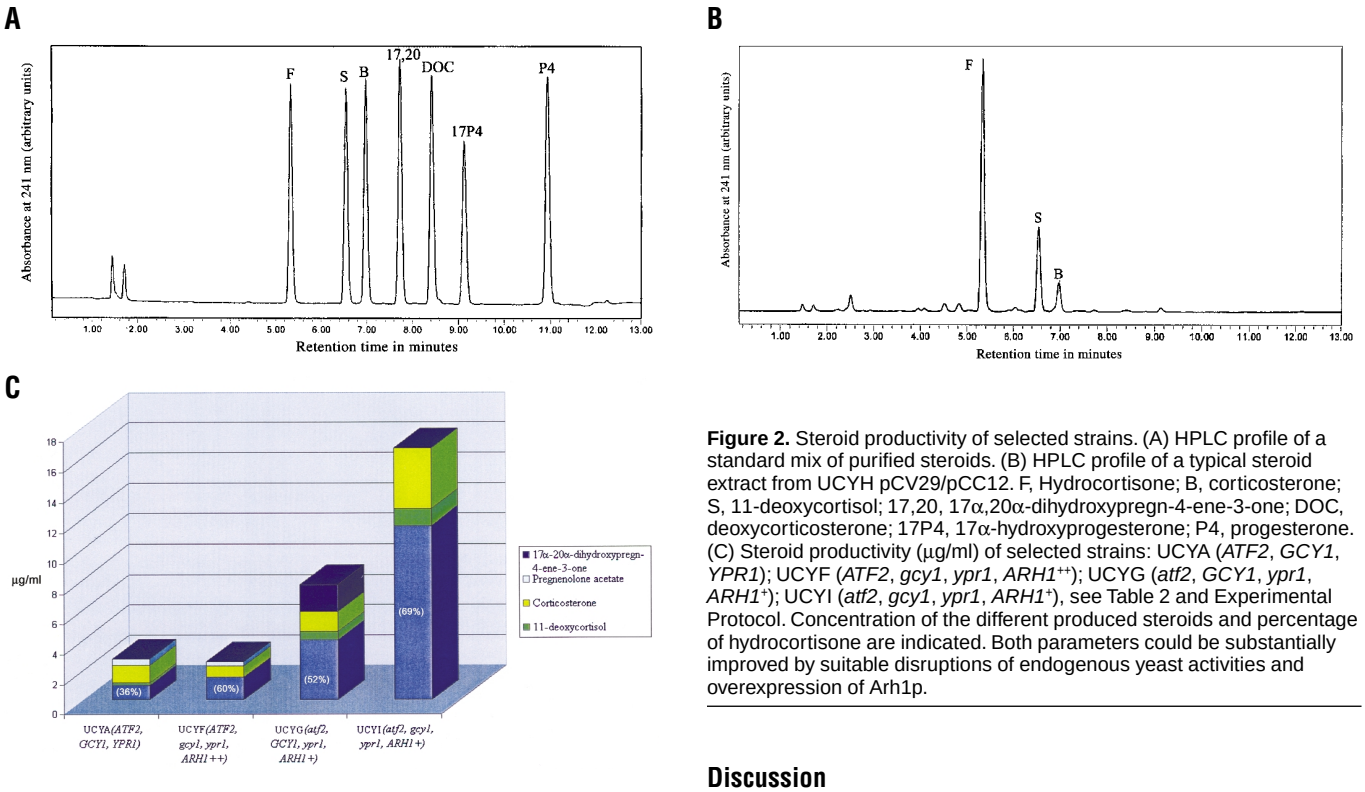

Figure 2. Steroid productivity of selected strains. (A) HPLC profile of a standard mix of purified steroids. (B) HPLC profile of a typical steroid extract from UCYH pCV29/pCC12. F, Hydrocortisone; B, corticosterone; S, 11-deoxycortisol; 17,20, 17α,20α-dihydroxypregn-4-ene-3-one; DOC, deoxycorticosterone; 17P4, 17α-hydroxyprogesterone; P4, progesterone. (C) Steroid productivity (µg/ml) of selected strains: UCYA (*ATF2*, *GCY1*, *YPR1*); UCYF (*ATF2*, *gcy1*, *ypr1*, *ARH1*$^{++}$); UCYG (*atf2*, *GCY1*, *ypr1*, *ARH1*$^{+}$); UCYI (*atf2*, *gcy1*, *ypr1*, *ARH1*$^{+}$), see Table 2 and Experimental Protocol. Concentration of the different produced steroids and percentage of hydrocortisone are indicated. Both parameters could be substantially improved by suitable disruptions of endogenous yeast activities and overexpression of Arh1p.

Hydrocortisone content improvement: Arh1p overexpression increases CYP11B1 activity. Arh1p, an essential yeast protein[7,13], has been shown to supply electrons to mammalian mitochondrial CYP11B1 *in vivo*, via mammalian ADX targeted to yeast mitochondria[6]. The accumulation by certain strains of detectable amounts of 11-deoxycortisol (Table 2) suggested that the flux of electrons from NADPH to CYP11B1 via ADX and Arh1p was limiting. Thus, Arh1p was overexpressed using two different promoters in the most elaborate strain, UCYD, yielding strains UCYE and UCYF. After plasmid transformation, the productivities of UCYD, UCYE, and UCYF were compared (Table 2). Increasing Arh1p synthesis using a stronger promoter appeared more favorable for the terminal part of the pathway. Production of hydrocortisone increased from 26% to 60% of the total steroids in the latter case as a result of a lower 11-deoxycortisol content.

Further productivity improvements: Atf2p activity has a critical control function in steroid production. To further improve the overall flux to hydrocortisone, the *ATF2* gene responsible for both pregnenolone acetylation[8] and prevention of further steroid metabolism was disrupted in strains UCYB, UCYC, UCYE, and UCYF, giving rise to UCYH, UCYG, UCYI, and UCYJ, respectively. These *atf2*-Δ strains were transformed by pCV29 and pCC12 for reconstitution of the complete pathway. Surprisingly, they showed a considerable increase (6- to 23-fold) in the yield of hydrocortisone, together with an increased accumulation of corticosterone, 11-deoxycortisol, and trace amounts of pregnenolone and progesterone (Table 2). In all strains, no deoxycorticosterone, 17α-hydroxyprogesterone, or 17α,20α-dihydroxypregn-4-ene-3-one (except for UCYG, which retains a functional *GCY1* gene) could be detected, indicating that the pathway was effectively equilibrated up to the last step of 11β-hydroxylation. Overall, the quantity of 3-keto steroids was increased 3- to 10-fold.

In conclusion, and as illustrated in Figure 2C for some selected strains, steroid production could be enhanced and channeled toward hydrocortisone through inactivation of side reactions (dependent on the *ATF2*, *GCY1*, and *YPR1* genes) and overexpression of the endogenous Arh1p.

Discussion

We report here on the production of hydrocortisone from a simple carbon source by recombinant *S. cerevisiae* strains. Because aerobic uptake of exogenous sterols by yeast is hindered, biosynthesis of the major sterol ergosterol was rerouted using a plant enzyme[3] to produce molecules resembling cholesterol. Major problems were encountered with the two mitochondrial steps (CYP11A1 and CYP11B1, Fig. 1). The former three-component side chain-cleaving reaction was reconstituted outside mitochondria using the mature forms of enzymes[3], whereas the latter reaction was targeted to mitochondria relying on a partially artificial electron transfer chain combining the natural yeast reductase Arh1p and the bovine ADX electron carrier[6].

In this work we identified two major unwanted side reactions: the esterification of pregnenolone by the *ATF2* gene product[8] and the 20-keto reduction of 17α-hydroxyprogesterone catalyzed by the concerted action of *GCY1* and *YPR1* gene products. The respective yeast genes were inactivated. Finally, the eight heterologous proteins (namely, mature forms of CYP11A1, ADX, and ADR; mitochondrial forms of ADX and CYP11B1; 3β-HSD, CYP17A1, and CYP21A1) of the mammalian pathway were simultaneously and functionally produced at suitable levels in the modified host. In the best case hydrocortisone was produced as the major steroid. This is the first successful transfer of a highly complex and mainly membrane-bound mammalian pathway into a microbial host. Five yeast genes were found to be decisive for the completion of the pathway: besides the aforementioned *ATF2*, *GCY1*, and *YPR1* possessing a negative impact, *NCP1* (NADPH-P450 reductase) and *ARH1* were indispensable for steroid production.

ATF2 disruption has both a positive effect, by preventing accumulation of dead-end pregnenolone acetate (12–24% of the steroid produced in wild-type *ATF2* strains), and a negative impact on steroid production, in that free pregnenolone inhibits the pathway through blockage of sterol biosynthesis[14]. Clearly, the positive effect was dominant in *atf2*-Δ strains in which hydrocortisone production was boosted 6- to 23-fold (Table 2 and Fig. 2C). Thus, the efficient conversion of pregnenolone into the less toxic progesterone might have a protective

Table 3. ***In vitro*** **and** ***in vivo*** **keto reductase activities of** ***Saccharomyces cerevisiae***

A. Keto-reductase specific activities of yeast cell-free extracts (in μM/min/mg proteins)[a]

Activities	FY 1679-28c (wt) (μM/min/mg)		TGY170 (*gcy1*::*URA3*) (μM/min/mg)		TGY197 (*gcy1*::*URA3*, *ypr1*::*LEU2*) (μM/min/mg)	
Substrates	**Glu**	**Gal**	**Glu**	**Gal**	**Glu**	**Gal**
Methylglyoxal	96	332	111	216	88	219
Glyceraldehyde	55	279	45	129	48	192
17α-Hydroxyprogesterone	0.03	0.6	0.005	0.008	<0.001	<0.001

B. Bioconversion of 17α-hydroxyprogesterone by different CYP21A1-expressing strains[b]

Products	FY1679-28C/ pTG10497 GCY1/YPR1 (% all ketosteroids)	TGY212#1 *ypr1-Δ* (% all ketosteroids)	TGY245#2D *gcy1-Δ*/ *ypr1-Δ* (% all ketosteroids)
17α-Hydroxyprogesterone	73.1	86.8	28.2
11-Deoxycortisol	18.7	1.5	71.7
17α,20α-Dihydroxypregn-4-ene-3-one	8.1	11.4	< 0.1

[a]Aldo-keto reductase was measured by monitoring the oxidation of NADPH; activity on 17α-hydroxyprogesterone was measured by quantification of 17α,20α-dihydroxypregn-4-ene-3-one (see Experimental Protocol). Carbon sources: Glu, glucose; Gal, galactose.
[b]17α-Hydroxyprogesterone (200 mg/L, 72 h of incubation, see Experimental Protocol) bioconversion into 11-deoxycortisol and 17α,20α-dihydroxypregn-4-ene-3-one was monitored using three different strains expressing CYP21A1. Results are expressed as a percentage of the sum of all ketosteroids detected. Only the relevant genotype is given.

effect. Progesterone was readily transformed by CYP17A1 into 17α-hydroxyprogesterone[15,16], which was further metabolized into 17α,20α-dihydroxypregn-4-ene-3-one except in the case where 20α-HSD was eliminated. Interestingly, the latter was only detected in UCYG (Tables 1 and 2), confirming that Gcy1p was mostly responsible for the 20-keto-reduction of 17α-hydroxyprogesterone. Ncp1p is known to supply electrons to at least five enzymes and was therefore expected to have a key function in the regulation of steroid biosynthesis. However, attempts to adjust its levels did not improve steroid biosynthesis and were frequently found to be deleterious (unpublished results). Arh1p is an essential reductase located in the inner mitochondrial membrane and is responsible for a crucial step in heme A biosynthesis[17]. We showed that Arh1p could efficiently replace ADR and make CYP11B1 functional *in vivo*[6]. Accumulation of 11-deoxycortisol was counteracted by overexpression of Arh1p in some of the strains (UCYE and UCYF)[18].

In conclusion, we have constructed yeast strains capable of self-sufficient production of hydrocortisone as the major steroid (up to 70% of total steroids) from glucose (or ethanol). In the best case 11-deoxycortisol and corticosterone were the only by-products. The strains described in this report represent a solid basis for the development of an environmentally friendly and low-cost industrial process to substitute for chemical approaches to the synthesis of corticoid drugs. Ongoing studies under optimized culture conditions have already demonstrated the potential for additional enhancements in productivity (data not shown). In addition, these strains constitute a solid model for analysing the crosstalk in mammalian steroid biosynthetic pathways.

Experimental protocol

Plasmid constructions. All the plasmids described (Table 4) can shuttle between *E. coli* and *S. cerevisiae*. They were constructed either by classical cloning procedures or by PCR and recombination in *E. coli*[19]. All expression cassettes contain a *PGK1* terminator. The cDNAs are from bovine origin except for CYP11B1 and CYP21A1, which are from human origin.

Strain constructions (see also Table 1). Yeast genetics and molecular biology were done according to published protocols[20]. TGY170 (FY 1679-28c, *gcy1*:*LEU2*) was constructed as described earlier with the disruption plasmid provided by W. Bandlow[21]. TGY197 (TGY170, *ypr1*:*URA3*) was generated by additional disruption of *YPR1*, using linear pTG12011. Strains possessing all the genetic material for hydrocortisone production were named UCY (Universal Corticoid Yeast). Except for UCYA, which is an isogenic derivative of CDR06, all the other UCYs strains (UCYD excluded) were transformed spores of the diploid strain SB14 obtained from crossing of CDR07 with TGY260-A. CDR06 and CDR07 are spores from the diploid strain obtained by crossing of FY1679-18b (ref. 22) with CA10 (ref. 3). TGY260-A derived from FY1679-18b MATa[22], where the two genes responsible for 20α-dehydrogenation of 17α-hydroxyprogesterone (i.e., *GCY1* and *YPR1*) were replaced by two expression cassettes for CYP21A1, and a second *ARH1* gene copy was added. First, *YPR1* was disrupted by the *URA3* gene using linear pTG12011. One clone (TGY195#4) was selected for its reduced capacity to transform 17α-hydroxyprogesterone into 17α,20α-dihydroxypregn-4-ene-3-one, which was tested as described[6]. In TGY195#4, the *URA3* gene was replaced using the *Not*I-digested pTG12045 and YRp7 (ref. 23). After selection of trp$^-$ and ura$^-$ colonies, the activity of the CYP21A1 cDNA was verified by bioconversion during 24 h of 17α-hydroxyprogesterone exposure as described[6,16]. One clone, TGY212#1, was shown to be capable of converting 17α-hydroxyprogesterone into 11-deoxycortisol with a minor production of 17α,20α-dihydroxypregn-4-ene-3-one. As for *YPR1*, *GCY1* disruption was achieved in two steps. Strain TGY212#1 was sequentially transformed with linearized pTG12010 and pTG12086, yielding strains TGY243#1 and TGY245#2D, respectively. Among ten clones, the latter gave the best conversion results, producing 11-deoxycortisol from 17α-hydroxyprogesterone in the absence of 17α,20α-dihydroxypregn-4-ene-3-one. This clone was selected for further transformation with pTG12048. Leu$^+$ colonies were selected, and the presence of an extra copy of *ARH1* was verified by PCR.

TGY260-A was crossed with CDR07, yielding diploid strain SB14. Spores from SB14 were selected on three criteria: auxotrophic markers, presence of the CYP21A1 cDNA, and resistance to nystatin. Spores that contained an extra copy of the *ARH1* gene (Leu$^+$, PCR), the CYP21A1 cDNA (PCR) and that produced Δ7-reduced sterol (Ade$^-$ and nystatin resistant[3]) were selected. Two spore clones, YCC4 and YCC5, were shown to produce ergosta-5-eneol and ergosta-5,22-dieneol as major sterols. Moreover, they were capable of converting 17α-hydroxyprogesterone into 11-deoxycortisol, producing, respectively, 120 and 42 μg/ml of 11-deoxycortisol starting from 300 μg/ml of 17α-hydroxyprogesterone in 72 h at 29 °C. YCC4 and YCC5 were sequentially transformed with linearized plasmids pLIP5 and pTG12093 (Table 4), selecting first for Trp$^+$ and then for His$^+$ colonies. Presence of a functional CYP17A1 cDNA was verified by PCR and by conversion of progesterone into 17α-hydroxyprogesterone as described[16]. Presence of the ADX cDNA was verified by PCR; the ADX protein was detected by western blot as described[6]. UCYB and UCYC were selected from these sequential transformations of YCC5 and YCC4, respectively.

CDR06 (Table 1) was submitted to the same successive rounds of cassette integration as YCC4 and YCC5, and was ultimately transformed using linear pTG12048 (for expression of *ARH1*) as described for TGY245. The presence of an extra *ARH1* expression cassette was verified by PCR spanning the *CYC1* promoter and the *ARH1* coding sequence. One clone, named UCYA, positive by PCR and having the desired auxotrophic profile, was taken for further study. In the UCY strains, the presence of CYP21A1 cassettes in *GCY1* and *YPR1* loci was verified using PCR in view of obtaining all three possible disruption combinations (Table 1). To obtain a strain devoid of both *GCY1* and *YPR1* genes, UCYC was crossed with TGY245#2D, and the resulting diploid YSA2 was sporulated. A set of spores complying with the same criteria as given earlier was chosen; among them, UCYD, having both *YPR1* and *GCY1* genes inactivated by CYP21A1 expression cassettes, was selected for further transformation. To overexpress Arh1p, UCYD was trans-

Table 4. Description of the plasmids used in this study

Name	Gene(s) disrupted or expressed[a]	Plasmid status	References	Marker
pTG12010	*gcy1*:*URA3*	Integration	pUC19 (ref. 26)	*URA3*
pTG12045	*gcy1*:P*TDH3*-CYP21A1	Integration	pTG12010	None
pTG12011	*ypr1*:*URA3*	Integration	pPOLYIII (ref. 27)	*URA3*
pTG12086	*ypr1*:P*TEF1*-CYP21A1	Integration	pTG12011	None
pAM1	None	ARS CENVI Replicon	pFL38C2 (ref. 28)	*ADE2*
pAM3	*ATF2*	ARS CENVI Replicon	pAM1	*ADE2*
pCV38	*atf2*:G418^R	Integration	pAM3, pFA6a KanMX4 (ref. 24)	G418 ^{R}
pLIP5	P*TEF1*-CYP17A1	Integration	pFL45S (ref. 28)	*TRP1*
pTG12093	P*TDH3*-COX6$_{pre}$::matADX	Integration	pUC19HIS3 (ref. 3)	*HIS3*
pTG12048	P*CYC1*-ARH1	Integration	pTG10953 (ref. 29), pFL26CD (ref. 3)	*LEU2*
pTG12050	P*TEF1*-ARH1	Integration	pTG10953, pFL26CD	*LEU2*
pCV29	P*CYC1*-COX6$_{pre}$::CYP11B1 P*GAL10/CYC1*-matCYP11A1 P*GAL10/CYC1*-matADX	2μ Replicon	pCD63 (ref. 3)	*URA3*
pCC12	P*TEF1*-matADR, P*TDH3*-3βHSD	ARS CENVI Replicon	pAM1	*ADE2*
pCC22	*Idem* pCC12+P*TDH3*-CYP21A1	ARS CENVI Replicon	pAM1	*ADE2*
pTG10497	P*TEF1*-CYP21A1	ARS CENVI Replicon	pTG10434 (ref. 30)	*LEU2*

[a]The prefix "mat" represents the mature form of the protein preceded by a methionine codon. The notation COX6$_{pre}$ represents the presequence of the cytochrome c oxidase subunit VI.

formed with linear pTG12048 or pTG12050. Two Leu$^+$ clones, named UCYE and UCYF, with an extra *ARH1* copy, were chosen (Table 1). To suppress formation of pregnenolone acetate in UCYB, UCYC, UCYE, and UCYF, *ATF2* was disrupted using linear pCV38. Gentamicin-resistant colonies[24] were isolated. *ATF2* gene activity was verified by conversion of pregnenolone into pregnenolone acetate[8].

Bioconversion and biosynthesis analysis. α-Keto reductase activity was measured in cell-free yeast extracts at 30 °C by monitoring by RP-HPLC[6] at 340 nm the oxidation of 1 mM NADPH in the presence of 20 mM methylglyoxal or glyceraldehydes or 20 μM 17α-hydroxyprogesterone in 50 mM potassium phosphate buffer, pH 7.4. Steroid reduction was quantified by RP-HPLC[6]. After 30 min at 30 °C, 2 ml of dichloromethane was added to stop the reaction. N_2-dried extracts of the organic phase were dissolved in acetonitrile and analyzed at 240 nm by RP-HPLC[6]. Steroids were eluted isocratically with 45% aqueous acetonitrile at a flow rate of 1 ml/min at 45 °C. *In vivo* conversion of 17α-hydroxyprogesterone by strains FY16796-28C/pTG10497, TGY212, and TGY245-2D was evaluated in complete synthetic medium[25] supplemented with 2% glucose, 1% wt/vol casaminoacids containing 200 μg/ml 17α-hydroxyprogesterone from a stock solution dissolved in a tergitol–ethanol solution at a 50:50 ratio (vol/vol). Cells were suspended in fresh medium at A_{600nm} of 10, and bioconversion occurred for 72 h at 29 °C. 17α,20α-dihydroxypregn-4-ene-3-one was quantified by RP-HPLC. To test steroid biosynthesis, 5–10 isolates of each of the transformants (UCYA pCV29/pCC22, UCYB to UCYJ pCV29/pCC12) were grown as described earlier for 24 h. Starting from this culture, the strains were cultivated from an A_{600nm} of 0.5 with 2% ethanol and 0.1% glucose as a carbon source for 172 h (with a 2% vol/vol ethanol addition at 72 h). All the strains showed the same growth kinetics, reaching an A_{600nm} close to 50 after 172 h. Extractions were done as described[6], except that 0.5 ml was extracted twice with 2 ml of dichloroethane. Half of the dry matter was resuspended in 100 μl dichloroethane, and 3 μl was analyzed by gas chromatography[3]. The other half was resuspended in 100 μl water–acetonitrile (1:1). Samples (20 μl) were analyzed by RP-HPLC[16], except that an X-Terra C18 column was used together with an acetonitrile:water gradient. The identity of each steroid was assessed by comparison with the retention time of pure compounds and confirmed by mass spectrometry analysis. Extractions were made in triplicates and results expressed as the mean of three extractions except for acetyl pregnenolone.

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Competing interests statement

The authors declare that they have no competing financial interests.

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