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BIOCONVERSION OF STEROIDS BY *COCHLIOBOLUS LUNATUS*—II. 11β-HYDROXYLATION OF 17α,21-DIHYDROXYPREGNA-1,4-DIENE-3,20-DIONE 17-ACETATE IN DEPENDENCE OF THE INDUCER STRUCTURE

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Summary—The 11β-hydroxylase of the filamentous fungus *Cochliobolus lunatus* m 118 was induced with the substrate 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate (11β-deoxyprednisolone 17-acetate) itself, substrate analogues, different pregnane compounds, sterols, intermediates of microbial sterol side-chain degradation or bile acids, together with 24 different steroids in a standardized test system. The resulting 11β-hydroxylation rate, leading to prednisolone 17-acetate and prednisolone, respectively, was determined and compared with the hydroxylation rate of non-induced cultures. The transformation yield strongly depended on the inducer structure. The microbial sterol side-chain degradation intermediates (20S)-20-hydroxymethylpregn-4-en-3-one and the corresponding pregna-1,4-diene compound caused the highest induction effects (induction factors 5.1 and 4.9, respectively). The metabolism of (20S)-20-hydroxymethylpregna-1,4-dien-3-one during the cultivation was elucidated. The induction effect decreased with the rising oxidation of the inducer. The significant increase of the 11β-hydroxylation rate of 1-dehydro-pregnane substrates by specific induction allows alternative pathways to glucocorticoid partial syntheses.

INTRODUCTION

The 11α-hydroxylases of *Aspergillus ochraceus*, *Rhizopus nigricans* and *Rhizopus arrhizus* [1–5] as well as the 11β-hydroxylases of *Curvularia lunata* [6, 7] and *Cochliobolus lunatus* [8, 9] have been characterized as inducible enzymes. The transformation yield of *Rhizopus nigricans* was found to be dependent on the induction conditions and the chemical structure of the inducer. Deoxycorticosterone and testosterone, steroids with a higher polarity at C-17, are poorer substrates for the hydroxylation but induced more enzyme compared to progesterone [5]. In *Cochliobolus lunatus* 21-hydroxyprogesterone (deoxycorticosterone) was also found to be the best inducer for the 11β-hydroxylation of progesterone [9].

In our studies we used this 11β-hydroxylating microorganism which was also found to hydroxylate steroids with a pregna-1,4-diene structure [10]. The enzyme system was characterized by examining cell-free extracts [11]. Steroids with 1-double bond, e.g. androsta-1,4-diene-3,17-dione (ADD) may be favourable precursors in glucocorticoid partial syntheses because the therapeutically important 1-double bond is already present in this molecule. On the other hand the 1-double bond hinders the microbial 11β-hydroxylation. Therefore we examined the 11β-hydroxylation of 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate, which could be a suitable precursor compound for the industrial preparation of prednisolone, in the presence of different steroid inducers.

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EXPERIMENTAL

Microbial strain

Cochliobolus lunatus m 118 was obtained from the strain collection of the Friedrich-Schiller-University of Jena, Freiherr-von-Stein-Allee 2, 0-5300 Weimar. It was selected from 54 microorganisms as a suitable strain for the 11β-hydroxylation of pregna-1,4-diene compounds [10]. In 1964 Nelson described the Asci

of this filamentous fungus and classified it taxonomically as the perfect state of *Curvularia lunata* which was related to the fungi imperfecti up to this date [12, 13].

The fungus was maintained on oatmeal agar slants consisting of 1.5% oatmeal (Pflug, Dippoldiswalde), 0.225% $CaCl_2$, 3% agar (Difco, Detroit), *aqua dest.*, pH 4.7–5.0. Agar slants were incubated for 11 days at 28°C and used to inoculate the precultures. Stock cultures were stored at 4°C and transferred to fresh medium after 6 months.

Steroids

Prednisolone, 17α,21-dihydroxypregn-4-ene-3,20-dione and 17α,21-dihydroxypregna-1,4-diene-3,20-dione were purchased from Serva (Heidelberg), deoxycholic acid from Reanal (Budapest) and cholic and lithocholic acid from Merck (Darmstadt). 17α,21-Dihydroxypregna-1,4-dien-20-one-3-carboxymethoxime and 17α-nitroxy-21-hydroxypregna-1,4-diene-3,20-dione were generous gifts from Dr Megges (ZIM, Berlin-Buch). 17α,21-Dihydroxypregna-1,4-diene-3,20-dione 17-acetate, 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-benzoate, 17α,21-dihydroxypregna-1,4-diene-3,20-dione 21-formiate, 17α,21-dihydroxypregna-1,4-diene-3,20-dione 21-methylcarbonate, 17α,21-(1-ethoxy-1-ethylidendioxy)-pregna-1,4-diene-3,20-dione, 17α,21-(1-methoxy-1-phenylmethylidendioxy)-pregna-1,4-diene-3,20-dione and 17α-ethinyl-17β-hydroxyandrosta-1,4-dien-3-one were synthesized at Jenapharm. β-Sitosterol was obtained from Farmos (Finland). Androst-4-ene-3,17-dione (AD), 9α-hydroxyandrost-4-ene-3,17-dione (9α-OH-AD), ADD, (20S)-20-hydroxymethylpregn-4-en-3-one, (20S)-20-hydroxymethylpregna-1,4-dien-3-one, (20S)-20-carboxypregn-4-en-3-one, (20S)-20-carboxypregna-1,4-dien-3-one and (20S)-20-carbomethoxypregna-1,4-dien-3-one were isolated and purified as microbial side-chain degradation products of β-sitosterol in our laboratories.

Standardized shaking culture test

Mature oatmeal agar slants were used to inoculate 500 ml culture flasks containing 100 ml of medium I consisting of 3.0% glucose (Merck), 0.5% lyophilized corn steep (Maisan, Barby), 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4 \times 7\ H_2O$, 0.002% $FeSO_4 \times 7\ H_2O$, 0.05% KCl, and *aqua dest.*, pH 6.5–6.7. The culture was shaken on a rotary shaker (180 strokes/min) for 60 h at 25°C. Ten millilitres of this preculture were used to inoculate 500 ml culture flasks filled with 100 ml of medium II consisting of 1.0% glucose, 1.0% lyophilized corn steep, 5 ml corn germ oil, and *aqua dest.*, pH 6.5–6.7. After 24 h of shaking at 28°C 500 ml culture flasks filled with 50 ml of main culture medium containing 0.7% glucose, 3% lyophilized corn steep, 0.075% KH_2PO_4, 0.15% $MgSO_4 \times 7\ H_2O$, 0.015% $FeSO_4 \times 7\ H_2O$, 0.015% $MnCl_2$, 0.005% $ZnSO_4 \times 7\ H_2O$, 0.1% $CaCl_2 \times 6\ H_2O$, and *aqua dest.*, pH 6.0 were inoculated with 5 ml of the second preculture. The inducer was then added as a solution in methanol or acetone (0.15 g/l; 0.2% v/v organic solvent). Non-induced cultures were treated with the organic solvent only. After 6 h of shaking at 28°C 1.5 g/l 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate were added as a methanolic solution (final concentration of the organic solvents <2% v/v). The incubation on the rotary shaker was continued for another 48 h at 28°C. Each experiment was performed with at least 3 parallels.

Determination of the hydroxylation activity

After 2 days of incubation samples were completely extracted with ethyl acetate and evaporated to dryness. The residue was dissolved in a definite volume of acetone and applied to TLC-plates (Silufol UV 254, 200 × 200 mm, Kavalier, Czechoslovakia). A fresh mixture of chloroform–methanol–cyclohexane–ethyl acetate (92:8:25:25, by vol) was used as the solvent system.

Spots were labelled under u.v.-light at 254 nm, stamped and eluted with ethanol for spectrometrical analysis. The extinction was measured in a spectrophotometer (VSU 2-P, Carl-Zeiss-Jena) at a wavelength of 242 nm. Finally values were corrected by blanks, and the steroid concentration was calculated via calibration curves.

RESULTS

Inducer screening

The amount of 11β-hydroxylated products (prednisolone 17-acetate and predisolone) was compared between induced and non-induced cultures. In non-induced cultures 0.687 mM ± 0.013 (mean of 9 determinations ± SD) of the substrate 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate were 11β-hydroxylated.

Table 1. Induction effect of the substrate (**1**) and of substrate analogues (**2–12**) on the microbial 11β-hydroxylation of 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate

Inducer	11β-Hydroxylated product (mM ± SD)	Induction factor	*n*
—	0.622 ± 0.152	1.00	3
1	1.088 ± 0.114	1.75	3
2	1.120 ± 0.210	1.80	3
3	1.088 ± 0.096	1.75	3
4	0.778 ± 0.087	1.25	3
5	0.964 ± 0.302	1.55	3
6	0.902 ± 0.224	1.45	3
7	1.244 ± 0.319	2.00	3
8	1.026 ± 0.256	1.65	3
9	0.902 ± 0.157	1.45	3
10	0.684 ± 0.125	1.10	3
11	0.529 ± 0.180	0.85	3
12	2.787 ± 0.350	4.48	5

The induction factor shows the relation of product yield between induced and non-induced cultures. Inducers: **1** = 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate, **2** = 17α,21-dihydroxypregna-1,4-diene-3,20-dione 21-formiate, **3** = 17α,21-dihydroxypregna-1,4-diene-3,20-dione 21-methyl-carbonate, **4** = 17α-nitroxy-21-hydroxypregna-1,4-diene-3,20-dione 21-ethylcarbonate, **5** = 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-benzoate, **6** = 17α,21-(1-methoxy-1-phenylmethylidendioxy)-pregna-1,4-diene-3,20-dione, **7** = 17α,21-(1-ethoxy-1-methylidendioxy)-pregna-1,4-diene-3,20-dione, **8** = 17α,21-dihydroxypregna-1,4-diene-3,20-one-3-carboxymethoxime, **9** = 17α,21-dihydroxypregna-1,4-diene-3,20-dione, **10** = 17α-ethinyl-17β-hydroxyandrosta-1,4-dien-3-one, **11** = 17α,21-dihydroxypregn-4-ene-3,20-dione, and **12** = progesterone.

Table 2. Induction effect of β-sitosterol (**13**), different microbial sterol side-chain degradation intermediates (**14–21**) and bile acids (**22–24**) on the microbial 11β-hydroxylation of 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate

Inducer	11β-Hydroxylated product (mM ± SD)	Induction factor	*n*
—	0.711 ± 0.124	1.00	3
13	0.747 ± 0.256	1.05	3
14	1.742 ± 0.321	2.45	3
15	0.995 ± 0.140	1.40	3
16	2.346 ± 0.420	3.30	3
17	2.417 ± 0.482	3.40	3
18	2.133 ± 0.387	3.00	3
19	1.102 ± 0.285	1.55	3
20	3.662 ± 0.572	5.15	5
21	3.484 ± 0.350	4.90	5
22	0.675 ± 0.105	0.95	3
23	0.818 ± 0.056	1.15	3
24	0.924 ± 0.082	1.30	3

For definition of the induction factor see Table 1. Inducers: **13** = β-sitosterol, **14** = AD, **15** = 9α-OH-AD, **16** = ADD, **17** = (20S)-20-carboxypregn-4-en-3-one, **18** = (20S)-20-carboxypregna-1,4-dien-3-one, **19** = (20S)-20-carbomethoxypregna-1,4-dien-3-one, **20** = (20S)-20-hydroxymethylpregn-4-en-3-one, **21** = (20S)-20-hydroxymethylpregna-1,4-dien-3-one, **22** = cholic acid, **23** = deoxycholic acid, and **24** = lithocholic acid.

The induction effect of different steroidal compounds is presented as the induction factor which is defined as the relation of the yield of 11β-hydroxylated product with inducer to the yield of 11β-hydroxylated product without inducer. The results are shown in Tables 1 and 2.

Inducer conversion

The inducer (20S)-20-hydroxymethylpregna-1,4-dien-3-one was transformed during the fermentation. The metabolites were isolated by preparative TLC and determined by mass-, NMR- and IR-spectroscopy [15]. Within the first hours of the transformation process the inducer was 11β-hydroxylated. After 20 h of fermentation the 11β,15β-dihydroxy compound occurred which was further transformed into 11-oxo-15β-hydroxy-(20S)-20-hydroxymethyl-1,4-pregnadien-3-one. The metabolism scheme is shown in Fig. 1. The inducer metabolites were tested themselves on their induction effects using the standardized shaking culture test. The results are shown in Table 3.

DISCUSSION

From 24 tested steroidal compounds 18 caused no, or only a low, induction effect; e.g. the substrate 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate itself (**1**), the intermediates of the chemical substrate synthesis as well as most of the substrate analogues (**2–11**), β-sitosterol (**13**), and the bile acids (**22–24**). Induction with the microbial side-chain degradation intermediates ADD, (20S)-20-carboxypregn-4-en-3-one or the corresponding 1,4-diene compound (**16–18**) increased the 11β-hydroxylation yield more than three times. By induction

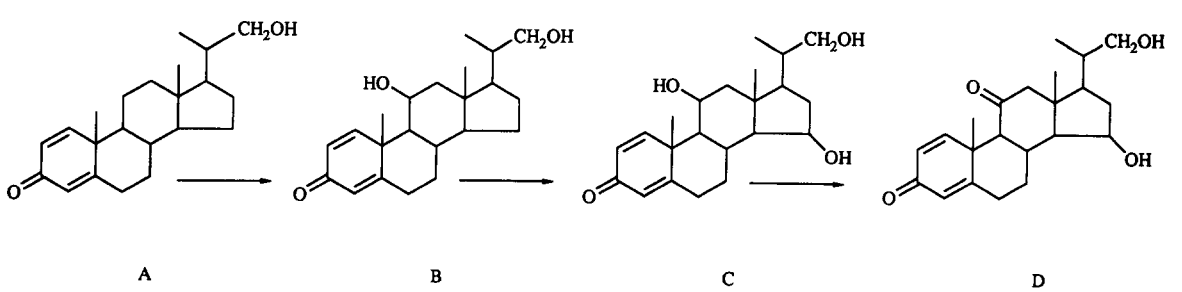

Fig. 1. Transformation of the inducer (20S)-20-hydroxymethylpregna-1,4-dien-3-one during the cultivation. **A** = (20S)-20-hydroxymethylpregna-1,4-dien-3-one, **B** = 11β-hydroxy-(20S)-20-hydroxymethylpregna-1,4-dien-3-one, **C** = 11β,15β-dihydroxy-(20S)-20-hydroxymethylpregna-1,4-dien-3-one, and **D** = 11-oxo-15β-hydroxy-(20S)-20-hydroxymethylpregna-1,4-dien-3-one.

Table 3. Induction effect of (20S)-20-hydroxymethylpregna-1,4-dien-3-one (A) and its metabolites (B–D) on the microbial 11β-hydroxylation of 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate

Inducer	11β-Hydroxylated product (mM ± SD)	Induction factor	n
—	0.727 ± 0.114	1.00	5
A	3.671 ± 0.355	5.05	5
B	2.726 ± 0.250	3.75	5
C	0.364 ± 0.036	0.50	5
D	0.327 ± 0.055	0.45	5

For explanation of the letters see legend to Fig. 1.

with progesterone (**12**), (20S)-20-hydroxymethylpregn-4-en-3-one (**20**) or (20S)-20-hydroxymethylpregna-1,4-dien-3-one (**21**) the transformation yield was increased up to about five times. In these cultures the substrate was nearly completely 11β-hydroxylated. The induction effect of (20S)-20-hydroxymethylpregna-1,4-dien-3-one decreased as metabolism proceeded. The 11β-hydroxy metabolite even caused a lowered but significant increase of the 11β-hydroxylation rate while the 11β,15β-dihydroxy- and the 11-oxo-15-hydroxy-compound caused no further induction.

All these results show that in *Cochliobolus lunatus* the induction effect strongly depends on the structure of the inducer and is especially influenced by C-17 substituents. C-22 steroids which occur as intermediates during the microbial side-chain degradation of β-sitosterol are good inducers of the 11β-hydroxylase in this filamentous fungus. There is a strong structural similarity in the side-chain between (20S)-20-hydroxymethylpregna-1,4-dien-3-one and 21-hydroxyprogesterone which was found to be a good inducer of the 11β-hydroxylation of progesterone with *Cochliobolus lunatus* [9]. Additional hydroxylation and other oxidative reactions at the steroid skeleton lead to compounds with a decreased induction effect.

The significant increase of the microbial 11β-hydroxylation rate by induction with (20S)-20-hydroxymethylpregna-1,4-dien-3-one using 17α,21-dihydroxypregna-1,4-diene-3,20-dione 17-acetate as substrate allows the efficient production of glucocorticoids from ADD. Both microbial and chemical procedures have already been developed on a technical scale [8, 16–18]. ADD is favourably obtained via side-chain degradation of β-sitosterol by mycobacteria [19]. (20S)-20-Hydroxymethylpregna-1,4-dien-3-one as a by-product of this microbial fermentation step is easily separatable by Ca-precipitation [14]. The advantage of this alternative pathway of glucocorticoid synthesis consists in avoiding the microbial 1-dehydrogenation as the last fermentation step in usual glucocorticoid partial syntheses.

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