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Biotransformation of Cortexolone to Hydrocortisone by Molds Using a Rapid Color-Development Assay[1]

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Abstract—The capacity of 22 molds for 11β-hydroxylation of cortexolone (Reichstein's compound S) to hydrocortisone were assessed. The biotransformation capacity was compared for solid-state and submerged monocultures of molds that were otherwise under identical conditions. Thin-layer chromatography and a novel rapid color-development assay were used to qualitatively establish the ability of fungi to convert cortexolone to hydrocortisone. These assays were validated and supplemented with data from high-performance liquid chromatography to obtain quantitative information on biotransformation. Nearly all the fungi consumed a significant fraction of the cortexolone fed, but only four of them (i.e., two isolates of *Cunninghamella blakesleeana, C. echinulata*, and *Curvularia lunata*) yielded measurable quantities of hydrocortisone. Submerged cultures generally gave a significantly greater yield of hydrocortisone compared to equivalent solid-state cultures.

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Steroidal drugs are an important class of compounds that are widely used in health and fertility management. Enzymatic and microbial biotransformations are used to produce various steroidal drugs and drug intermediates. For example, biotransformations of steroidal precursors provide structures such as 11-α-hydroxyprogesterone, 16-α-hydroxyprogesterone, hydrocortisone, and prednisolone [1–4]. Sequential and mixed cultures of fungi and bacteria have been frequently used to produce prednisolone from cortexolone [5–10]. Cortexolone (17α,21-dihydroxy-4-pregnene-3,20-dione, $C_{21}O_4H_{30}$, or Reichstein's compound S) can be converted to hydrocortisone (11β,17α,21-trihydroxy-4-pregnene-3,20-diene, $C_{21}O_5H_{30}$, or Kendall's compound F) by 11β-hydroxylation (Fig. 1) [1, 7, 11–14]. This paper is concerned with the identification of biotransformation capacity in molds good for converting the readily available intermediate cortexolone to hydrocortisone, an anti-inflammatory drug.

The 11β-hydroxylation of cortexolone is one of the most important steroidal biotransformations and is used for producing cortisol and other therapeutic steroids [2, 15–17]. Certain fungi can hydroxylate cortexolone at both 11α- and 11β-positions, but other fungi hydroxylate exclusively at only one of these positions [18]. Biotransformations of cortexolone have been generally carried out with freely suspended fungal biomass [19, 20], immobilized mycelia [7, 13], and fungal spores [21, 22]. Fungal protoplasts and cell-free preparations have also been used for the biotransformation [23, 24]. Various microbial cortexolone hydroxylations to compounds other than hydrocortisone have been documented [18, 20, 24, 25].

Solid-state and submerged batch cultures of 22 molds were used to assess their capabilities for converting cortexolone to hydrocortisone. A color-reaction assay was devised to rapidly establish if any cortexolone had been converted to hydrocortisone.

MATERIALS AND METHODS

Microorganisms. The following mycelial fungi were used: two isolates of *Cunninghamella blakesleeana*; *C. echinulata*; *Curvularia lunata* ATCC 12017; two isolates of *Curvularia* sp.; six isolates of *Penicillium* sp.; two isolates of *Fusarium* sp.; two isolates of *Aspergillus* sp.; *Calcarisporium antibioticum*; *Cladosporium* sp.; *Mucor* sp.; *Acremonium* sp.; *Paecilomyces* sp.; and *Rhizopus* sp. The fungi were maintained on Sabouraud dextrose agar (**SDA**; Difco, USA) slants at 4°C.

Culture and biotransformation methodologies All microorganisms were grown as monocultures. The molds were subcultured on SDA Petri dishes at room temperature 28 ± 2°C for 72–120 h. A loopfull of the culture was then transferred to 50 ml of enriched growth medium held in a 250-ml shake flask (220 rpm). The enriched medium contained the following components (g/l): glucose, 70; peptone, 1.0; yeast extract, 2.0; *L*-asparagine, 0.7; nicotinic acid, $2\ 2 \times 10^{-4}$; and riboflavin, 1.13×10^{-3}. The pH was adjusted to 7.8 before autoclaving 121°C, 20 min. After 72 h of incubation, the contents of the flask were divided into two identical

[1] The text was submitted by the authors in English.

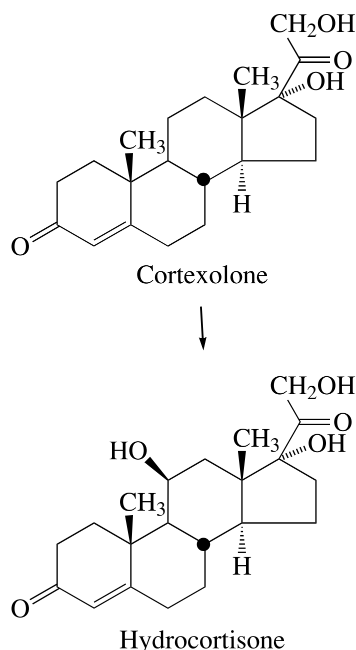

Fig. 1. 11β-Hydroxylation of cortexolone to hydrocortisone.

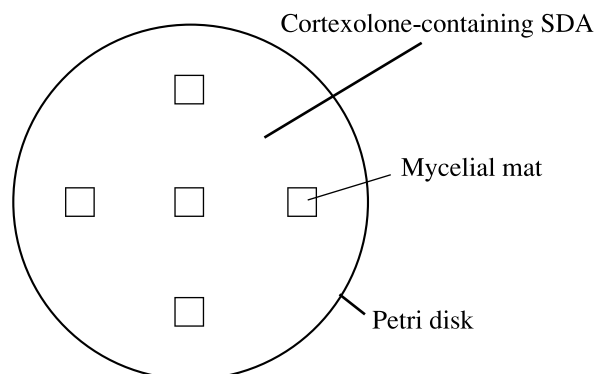

Fig. 2. Schematic placement of the five mycelial mats (5×5 mm) at inoculation on a Petri dish of cortexolone-containing SDA.

shake flasks (250 ml), and 24 ml of the aforementioned growth medium were added to each flask. The two flasks provided duplicate data. In all cases, the culture flasks were aerated by sparging sterile air through the culture. After 48 h of incubation at 28 ± 2°C, 15 mg of cortexolone (Sigma, USA) dissolved in 1 ml of 95% ethanol was added to each flask. This provided the initial cortexolone concentration of 15 mg in the 50-ml final volume of the broth. Incubation continued for seven days. Samples (1 ml) were collected every day, including the first, for the next seven days after the addition of cortexolone. For use in subsequent analyses, each sample was extracted with 2 ml of chloroform.

Because the submerged culture procedure was cumbersome and required a 72-h-long intermediate shake-flask incubation step, a more efficient solid-state biotransformation method was assessed in parallel with the submerged culture already described. For solid-state biotransformation, the fungi were subcultured on SDA Petri dishes at room temperature for 72–120 h. Then, ten pieces (5×5 mm each) of the mycelium layer were cut from the hyphal mat and transferred to two fresh, identical, sterile Petri dishes (five pieces per dish, placed as shown in Fig. 2). These Petri dishes contained SDA mixed with cortexolone (15 mg per 50-ml SDA). Incubation continued for seven more days. During this period the fungal growth covered the entire surface of the agar. After incubation, the contents of the duplicate Petri dishes (15–25 g per dish) were harvested separately, weighed, finely ground with a spatula, and extracted with chloroform (15 ml). The extract was analyzed for cortexolone and hydrocortisone by the three methods detailed in the next section.

Analyses Thin-layer chromatography (**TLC**) and a rapid color-development assay were used for a qualitative determination of cortexolone and hydrocortisone. For TLC analyses, a 50 µl sample was spotted on the TLC plate (silica gel 60 mesh GF_{254}; Merck, Germany). The mobile phase was 40% acetone in chloroform. The spots were visualized under ultraviolet light (254 nm) (Camag UV Viewer, Switzerland). The approximate R_f values of pure cortexolone and hydrocortisone were 0.6 and 0.35, respectively.

For the rapid color-development assay, concentrated sulfuric acid was added to an equal volume of the filtered chloroform extract of the fermentation broth or the solid-state growth. Development of a pink color indicated the exclusive presence of cortexolone, i.e., no conversion to hydrocortisone. A yellow–green color indicated the exclusive presence of hydrocortisone, i.e., a total conversion of cortexolone. An orange color indicated a presence of both cortexolone and hydrocortisone in the extract. The qualitative indications of the color-development assay were confirmed by TLC and HPLC. The latter technique also provided quantitative data. The analytical standards of cortexolone and hydrocortisone for use in HPLC and TLC were purchased from Sigma (USA).

For HPLC analysis of cortexolone and hydrocortisone, the filtered chloroform extract (20 µl) of the sample was autoinjected into the HPLC (Thermoseparation Products, Spectra Systems AS 3000, Fremont, USA). A C_{18} reversed-phase column (Luna, 10 µm, 250×4.6 mm; Phenomenex, USA) was used. The UV detector was set to measure at 239 nm. The eluent was aqueous methanol (70% v/v methanol); the eluent flow rate was 1.0 ml/min. Analyses were performed at an ambient temperature. The retention times of cortexolone and hydrocortisone were 5 and 6 min, respectively.

Hydrocortisone yield and cortexolone remaining for different fungi and growth conditions*

Mold	Submerged culture method			Agar plate method		
	time at maximum yield, *d*	residual cortexolone, % ± SD	yield of hydrocortisone, % ± SD	time at measured yield, days	residual cortexolone, % ± SD	yield of hydrocortisone, % ± SD
Cunninghamella blakesleeana ATCC 8688 a	5	4.5 ± 0.2	40.8 ± 3.2	7	90.2 ± 42.7	53.5 ± 10.4
C. blakesleeana ATCC 8688 b	5	3.8 ± 0.7	42.4 ± 6.8	7	3.2 ± 0.2	8.1 ± 2.0
C. echinulata F 1307943	2	51.7 ± 2.4	21.5 ± 0.2	7	56.7 ± 1.4	5.9 ± 0.2
*Curvularia lunat*a ATCC 12017	7	12.7 ± 4.2	35.0 ± 6.8	7	33.8 ± 2.6	9.9 ± 0.9
C. sp. TISTR 3293	2	33.7 ± 12.4	n.d.	7	56.2 ± 14.9	n.d.
C. sp. TISTR 3289	2	50.3 ± 2.8	n.d.	7	4.0 ± 1.4	n.d.
Penicillium sp. F 61388	2	52.0 ± 1.4	n.d.	7	14.0 ± 0.0	n.d.
P. sp. F 613812	2	55.0 ± 6.1	n.d.	7	48.7 ± 0.8	n.d.
*P.*sp. F 70941	2	65.3 ± 3.8	n.d.	7	0.3 ± 0.1	n.d.
P. sp. F 879407	2	48.8 ± 6.4	n.d.	7	20.2 ± 11.1	n.d.
P. sp. F 879413	2	37.0 ± 6.1	n.d.	7	29.3 ± 10.4	n.d.
P. sp. F 104943	2	34.3 ± 4.7	n.d.	7	20.3 ± 3.8	n.d.
Fusarium sp. F 61387	2	84.3 ± 8.5	n.d.	7	34.8 ± 33.7	n.d.
F. sp. F 127948	2	64.3 ± 7.5	n.d.	7	41.5 ± 2.1	n.d.
Aspergillus sp. F 879404	2	60.0 ± 10.4	n.d.	7	43.2 ± 1.2	n.d.
A. sp. F 879408	2	56.0 ± 2.4	n.d.	7	29.5 ± 5.0	n.d.
*Calcarisporium antibioticu*m	2	40.0 ± 1.9	n.d.	7	35.0 ± 1.4	n.d.
Cladosporium sp. F 104944	2	48.0 ± 9.9	n.d.	7	78.3 ± 11.3	n.d.
Mucor sp. F 148943	2	42.7 ± 0.9	n.d.	7	69.5 ± 8.7	n.d.
Acremonium sp. F 53946	2	120.7 ± 12.3	n.d.	7	90.0 ± 2.4	n.d.
Paecilomyces sp. F 104949	2	102.3 ± 11.8	n.d.	7	38.7 ± 9.0	n.d.
Rhizopus sp. F 104943	2	81.3 ± 13.2	n.d.	7	44.7 ± 13.2	n.d.

* SD, standard deviation; n.d., not detected.

RESULTS AND DISCUSSION

The maximum yields of hydrocortisone from cortexolone obtained in submerged cultures of various fungi are shown in the table. For the solid-state cultures, measurements were only made on day seven and, therefore, the observed yield of hydrocortisone in the table is not necessarily the maximum yield that is attainable. The data on the fraction of the initial cortexolone remaining are also shown in the table.

The results in the table reveal several points. Only four of the 22 fungi tested could convert cortexolone to hydrocortisone. The fungi that were capable of this biotransformation did so both in submerged and solid-state cultures. The hydrocortisone yield in submerged cultures was generally better than in equivalent solid-state cultures, especially if the data are viewed in the context of the time it took to attain the various yields. Although most fungi did not produce detectable hydrocortisone, they nevertheless consumed cortexolone. In these cases, cortexolone may have been converted to some of its other known biotransformation products [18, 20, 24, 25]. The data of hydrocortisone yield in the table are based on the consumed fraction of cortexolone. In two cases, the measured residual fraction of cortexolone exceeded 100%. But this should be viewed in the context of a high standard deviation for those measurements.

As noted above, the submerged culture biotransformation required a total minimum time of >120 h to produce any results. In contrast, the solid-state culture

method required a minimum time of 72 h. This improved efficiency, made possible by the solid-state methodology, can be a significant advantage when screening many different microorganisms for the desired biotransformation capacity. The results proved that a microorganism capable of the desired conversion would typically do so both in submerged and solid-state cultures. Extraction of the liquid sample from a submerged culture was relatively easy compared to extraction of the Petri dish contents of the solid-state growth. However, both procedures appeared satisfactory for establishing the relative biotransformation capacity of various fungi.

Although solid-state culture may be good for quickly identifying the hydrocortisone producing fungi, the results suggest that submerged culture should be preferred for this biotransformation in production processes. However, this is not necessarily so, as neither of the culture methodologies were optimized to any extent. For at least some of biotransformation processes, solid-state culture can be superior to a submerged culture [26].

Microbial biotransformations with live cells can be influenced by factors such as the composition of the growth medium, the temperature, pH, and the concentration of dissolved oxygen. Although the influence of these factors on the 11β-hydroxylation was not investigated in this study, 11β-hydroxylation of cortexolone by *C. lunata* is known to be affected by the pH and dissolved oxygen concentration [14]. In addition, other microbial hydroxylations of cortexolone are known to be influenced by environmental and nutritional factors [9, 19, 27]. Consequently, the biotransformation capacity of the promising fungi identified in this study can potentially be enhanced significantly by optimizing the culture media and the environment.

A rapid screening of microorganisms for the ability to produce hydrocortisone from cortexolone cannot rely on the relatively slow HPLC analyses for measuring the substrate and the product. Colored compounds produced by reacting an analyte with a reagent can be used to detect and quantify the analyte. Reactions of steroids with sulfuric acid produce colored products [28] that can be the basis for rapidly detecting the presence of certain steroids. Our experiments showed that adding concentrated sulfuric acid to an equal volume of the analyte dissolved in chloroform produced pink, yellow–green, and orange colors when the analyte was pure cortexolone, pure hydrocortisone, and a mixture of the two compounds, respectively. This instantaneous qualitative color-reaction assay proved useful in quickly identifying the presence of hydrocortisone in culture samples.

Of the 22 fungi tested, all of them converted cortexolone to various unidentified products, but only four fungi transformed it to hydrocortisone. These fungi were the two isolates of *Cunninghamella blakesleeana* ATCC 8688, *C. echinulata*, and *Curvularia lunata* ATCC 12017. The fungi that produced hydrocortisone converted some of the cortexolone to other products. Solid-state cultures were relatively faster than submerged cultures in clearly identifying the hydrocortisone producing fungi; however, compared to submerged cultures, the hydrocortisone yield of solid-state fermentations was generally low.

The color-development assay provided an extremely rapid and reliable method for distinguishing hydrocortisone producing fungi from those that did not produce hydrocortisone. This assay is recommended for use in future screenings of microorganisms for biotransforming cortexolone to hydrocortisone.

REFERENCES

1. Sebek, O.K. and Perlman, D., *Microbial Technology*, Peppler, H.J. and Perlman, D., eds., 2nd ed., vol. 1, London: Academic, 1979.
2. Sedlaczek, L., *Crit. Rev. Biotechnol.*, 1988, vol. 7, pp. 187–236.
3. Manosroi, J., Abe, M., and Manosroi, A., *Bioresource Technol.*, 1999a, vol. 69, pp. 67–73.
4. Manosroi, J., Manosroi, A., Saraphanchotiwittaya, A., and Abe, M., *J. Chem. Technol. Biotechnol.*, 1999b, vol. 74, pp. 364–370.
5. Kimura, T., *On the Transformation of Reichstein's Substance S to Prednisolone with the Co-operative Action of Fungi and Bacteria*, *Osaka: Shionogi Research Laboratory,* 1962, vol. 12, pp. 180–185.
6. Ryu, D., Lee, B.K., Thoma, R.W., and Brown, W.E., *Biotechnol. Bioeng.*, 1969, vol. 11, pp. 1255–1270.
7. Mazumder, T.K., Sonomoto, K., Tanaka, A., and Fukui, S., *Appl. Microbiol. Biotechnol.*, 1985, vol. 21, pp. 154–161.
8. Ghanem, K.M. and Yusef, H.H., *Biomed. Lett.*, 1992a, vol. 47, pp. 201–209.
9. Ghanem, K.M. and Yusef, H.H., *Can. J. Microbiol.*, 1992b, vol. 38, pp. 753–757.
10. Ghanem, K.M., El-Aassar, S.A., and Yusef, H.H., *J. Chem. Technol. Biotechnol.*, 1992, vol. 54.
11. Shull, G.M., Kita, D.A., and Davisson, J.W., US Patent no. 2 658 023, 1953.
12. Shull, G.M. and Kita, D.A., *J. Am. Chem. Soc.*, 1955, vol. 77, pp. 763–764.
13. Sonomoto, K., Hoq, M.M., Tanaka, A., and Sukui, S., *Appl. Environ. Microbiol.*, 1983, vol. 45, pp.
14. Chen, K.C., and Wey, H.C., *Enzyme Microbiol. Technol.*, 1990, vol. 12, pp. 305–308.
15. Schmauder, H.P., Schlosser, D., Gunther, T., Hattenbach, A., Sauerstein, J., Jungnickel, F., and Augsten, R., *J. Basic Microbiol.*, 1991, vol. 31, pp. 454–477.
16. Mahato, S.B. and Garai, S., *Steroids.*, 1997, vol. 62, pp. 332–345.
17. Lisowska, K. and Dlugonski, J., *J. Basic Microbiol.*, 1999, vol. 39, pp. 117–125.
18. Clark, T.A., Maddox, I.S., and Chong, R., *Eur. J. Appl. Microbiol. Biotechnol.*, 1983, vol. 17, pp. 211–215.
19. Clark, T.A., Chong, R., and Maddox, I.S., *Appl. Microbiol. Biotechnol.*, 1985, vol. 21, pp. 132–134.

20. Sedlaczek, L., Jaworski, A., and Wilmanska, D., *Eur. J. Appl. Microbiol. Biotechnol.*, 1981, vol. 13, pp. 63–69.
21. Jaworski, A., Sedlaczek, L., Sasiak, A., and Dlugonski, J., *Eur. J. Appl. Microbiol. Biotechnol.*, 1982, vol. 16, pp. 63–69.
22. Horhold, C., Undisz, K., Groh, H., Sahm, R., Schade, W., and Komel, R., *J. Basic Microbiol.*, 1986, vol. 26, pp. 335–339.
23. Paraszkiewicz, K. and Dlugonski, J., *J. Biotechnol.*, 1998, vol. 65, pp. 217–224.
24. Klimova, L.I., Malanina, G.G., Filipenko, T.Y., and Turchin, K.F., *Khimiko-Farmatsevt. Zh.*, 1989, vol. 23, no. 9, pp. 1122–1124.
25. Garai, S. and Mahato, S.B., *Steroids.*, 1997, vol. 62, pp. 253–257.
26. *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*, Chisti, Y., Flickinger, M.C., and Drew, S.W., eds., New York: Wiley, 1999, vol. 5, pp. 2446–2462.
27. Clark, T.A., Chong, R., and Maddox, I.S., *Eur. J. Appl. Microbiol. Biotechnol.*, 1982, vol. 14.
28. Stevens, H.M., *Clarke's Isolation and Identification of Drugs (in Pharmaceuticals, Body Fluids, and Post-Mortem Material)*, 2nd ed., Moffat, A.C., Jackson, J.V., Moss, M.S., and Widdop, B., eds, London: The Pharmaceutical Society of Great Britain, 1986.