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Synthesis of the antibiotic cortalcerone from D-glucose using pyranose 2-oxidase and a novel fungal enzyme, aldos-2-ulose dehydratase

Kirston Koths, Robert Halenbeck and Margaret Moreland [1]

Cetus Corporation, Department of Protein Chemistry, 4560 Horton Street, Emeryville, California 94608 (USA)

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ABSTRACT

Using two enzymes purified from the white-rot fungus, *Polyporus obtusus*, 5% solutions of D-glucose have been quantitatively converted in vitro into D-*arabino*-hexos-2-ulose (D-glucosone) and subsequently into a compound having antimicrobial activity. The antibiotic has been shown by nuclear magnetic resonance and mass spectroscopy to be chemically identical to a previously described fungal metabolite known as cortalcerone. Based on kinetic analysis of the synthetic process, a pathway for the biosynthesis of cortalcerone is proposed, involving both chemical rearrangement and enzymically catalyzed steps. Two enzymes, pyranose 2-oxidase and a previously uncharacterized D-*arabino*-hexos-2-ulose-utilizing enzyme, may be sufficient for the biosynthesis of cortalcerone from glucose in vivo. The D-*arabino*-hexos-2-ulose-utilizing enzyme dehydrates certain aldosuloses and has been named aldos-2-ulose dehydratase. The enzyme, which appears to be a dimer of 95-kDa subunits, has been purified 450-fold. Additional properties of aldos-2-ulose dehydratase are described, including its apparent ability to catalyze two different steps in the proposed biosynthetic pathway for cortalcerone.

INTRODUCTION

We have characterized an apparent biosynthetic pathway for the production of the six-carbon antibiotic cortalcerone, a secondary metabolite first discovered by Baute and colleagues in the fungus *Corticium caeruleum* [1–3]. The existence of a related pathway in the fungus *Polyporus obtusus* became apparent when we used the fungal enzyme, pyranose 2-oxidase (EC 1.1.3.10) in vitro according to Liu [4] to oxidize D-glucose at the 2 position to yield D-*arabino*-hexos-2-ulose (also known as D-glucosone). During those experiments it was discovered that another enzyme, present as a contaminant in the pyranose 2-oxidase, was capable of converting the formed aldosulose to other products. We now report the purification and charac-

Correspondence to: Dr. K. Koths, Cetus Corporation, Department of Protein Chemistry, 4560 Horton Street, Emeryville, CA 94608, USA.
[1] Current address: Glycomed, Inc., 860 Atlantic Ave., Alameda, CA 94501, USA.

terization of that enzyme and the cortalcerone product (5,6-dihydro-6-hydroxy-5-oxo-2*H*-pyran-6-carboxaldehyde hydrate) resulting from its action.

Although the existence of D-*arabino*-hexos-2-ulose has been known for over a century [5], the compound has not been extensively studied because its yield in chemical synthesis is low and purification from byproducts is difficult [6]. As shown in this report, however, the enzymically catalyzed reactions for the production of D-*arabino*-hexos-2-ulose, D-*threo*-pentos-2-ulose, and cortalcerone in vitro can be driven essentially to completion at moderately high concentrations without formation of byproducts. Consequently, such reactions may be of use in industrial processes involving conversion of inexpensive glucose feedstocks into commodity or specialty chemicals of higher value. Recent interest in D-*arabino*-hexos-2-ulose itself has centered on its possible antiproliferative activity on human cancer cell lines [7], its formation following sterilization of food products by irradiation [8,9], and its use in the preparation of brain-imaging reagents [10].

MATERIALS AND METHODS *

Enzyme preparations used in the synthesis of D-arabino-*hexose-2-ulose.—(1) Repurified* Aspergillus niger *catalase.* Purified *A. niger* catalase from Fermco (Lot No. 4927, 154 000 U/g) was further purified to remove glucose 1-oxidase, which would generate an unwanted byproduct from glucose. One gram of the enzyme was bound to a CM-Sepharose column (5 × 12 cm, Pharmacia) and eluted with a 0–150 mM gradient of NaCl in 20 mM NaOAc, pH 4.7. The bulk of the catalase (monitored by absorption at 404 nm) separated from the glucose 1-oxidase activity (measured by an orthodianisidine assay of glucose-dependent peroxide generation). The repurified catalase had essentially no glucose-utilizing activity as measured by high-performance liquid chromatography of glucose treated with the enzyme.

(2) Partially purified pyranose 2-oxidase. Pyranose 2-oxidase (P2O) was partially purified from the mycelium obtained from a 1–100 L fermentation of *Polyporus obtusus* by precipitating it with PEG, as described by Ruelius [11]. Additional contaminants were removed by precipitating P2O from the solubilized pellet at pH 5.0 with acetic acid, and centrifuging at 10 K × *g* for 15 min. The product had a final specific activity of about 3.0 U/mg, whereas P2O was found to have a specific activity of about 8.5, indicating that a significant fraction of the material in the partially purified preparation was not P2O. Furthermore, unless special precautions were taken to prevent proteolysis, the procedure of Ruelius [11] resulted in a P2O preparation that had suffered at least two cleavages per subunit, yet retained activity.

(3) Highly purified pyranose 2-oxidase. A method for preparing nonproteolyzed

* Abbreviations used: P2O, pyranose 2-oxidase; PEG, polyethylene glycol; DEAE, diethylaminoethyl; CM, carboxymethyl; SDS, sodium dodecyl sulfate; TTC, triphenyltetrazolium chloride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid.

P. obtusus P2O that contains essentially no aldosulose-utilizing contaminants has been developed [12]. The purification involves PEG 4000 precipitation as well as DEAE-Sepharose and molecular-sieve chromatography. The final specific activity was 8.5 U/mg, as determined by measuring protein concentration using the method of Lowry [13], and enzyme units by following glucose-dependent peroxide formation using an orthodianisidine assay [11]. No contaminants were detectable by SDS-PAGE.

HPLC analysis of reaction products.—Chromatography was performed using a Waters μBondapack carbohydrate column eluted with 78% acetonitrile in 3 mM sodium phosphate, pH 6.0. The flow rate was 2.0 mL/min, and detection was by refractive index or by UV absorption at 192 nm. The chemical identity of the observed peaks was determined in part by comparing retention times with those of known standards.

Enzymic synthesis of pure, protein-free D-arabino-*hexos-2-ulose.*—To produce D-*arabino*-hexos-2-ulose at a purity of over 99% (as analyzed by UV spectroscopy or HPLC), highly purified enzymes such as those described above were required. With 0.92 U/mL of both pure P2O and repurified *A. niger* catalase, a 2% solution of D-glucose in 10 mM citrate, pH 5, was converted into D-*arabino*-hexos-2-ulose in 6 h at 25°. Catalase and P2O were removed by filtration through an Amicon PM30 filter at 4°. The final product was stored at 4° or frozen at −20°, under which conditions it was stable for at least one year.

Spectrophotometric assays for aldos-2-ulose dehydratase activity.—In a typical A_{265} assay, 0.8 mL of a 125 mM solution of D-*arabino*-hexos-2-ulose (having an initial absorbance of less than 0.15 at 265 nm) was incubated at 25° with 10 μg of partially purified aldos-2-ulose dehydratase and monitored continuously at 265 nm. Approximate initial rates were calculated from the change in absorbance between minutes one and two of the reaction. The A_{265} assay results was shown to vary linearly with enzyme concentration. Because of its speed, this assay was routinely used to locate the enzyme in column fractions. To utilize absorbance at 230 nm as an assay, the above protocol was modified by incubating substrate with the enzyme for 15 min prior to determining the rate of change of A_{230}. To maximize accuracy using either assay, the D-*arabino*-hexos-2-ulose concentration was kept relatively high, since the K_m for D-*arabino*-hexos-2-ulose (determined at pH 7.0 by the Lineweaver–Burk method, using the A_{265} assay) was found to be about 40 mM (data not shown).

Enzyme units for the aldos-2-ulose dehydratase activity were defined as μmol of cortalcerone produced per min at 25° in 0.1 M sodium phosphate buffer, pH 6.0. The relationship between absorbance at 230 nm and cortalcerone concentration was determined as follows: A freshly prepared 6% (w/v) solution of D-glucose was completely converted into a 6% solution of D-*arabino*-hexos-2-ulose, which has relatively little absorbance at 230 nm. This solution was diluted to 1.2 times its original volume with a solution of dehydratase and the reaction was allowed to proceed to 92% conversion. The resulting 4.6% cortalcerone solution had an

extinction coefficient at 230 nm of approximately 5200 L/mol · cm (compared to the literature value for cortalcerone of 8100 L/mol · cm obtained by Baute [19]).

Testing the aldosulose-derived product for antibiotic activity.—Immediately following their synthesis, equivalent concentrations of deproteinized D-*arabino*-hexos-2-ulose substrate and product were tested for antibiotic activity in two ways. First, 20 μL of a 290 mM, filter-sterilized solution of each compound was applied to a 6-mm filter disc and the discs were incubated on an agar plate seeded with *Bacillus subtilis, Serratia marcescens*, or *Staphylococcus aureus*. Plates were examined for zones of clearing following 24 h of incubation at 30°. Growth inhibition was also examined by testing various concentrations of the compounds for effects on the growth of *E. coli* strain K in 2× concentrated, pH 5.9 Luria broth [14]. Growth rates were assayed by increase in absorbance at 550 nm during incubation at 37°.

Chemical characterization of the aldosulose-derived product.—NMR spectra were recorded on a Varian FT 80A spectrometer (80 MHz for ^{1}H and 20 MHz for ^{13}C). Chemical shifts are reported in ppm from $(CH_3)_4Si$. IR of solid samples in KBr discs were obtained using a Perkin–Elmer 727B spectrometer. Chemical ionization mass spectra (isobutane as the reagent gas) were recorded on a Finnigan 4000 instrument. The sample, in water solution, was introduced through a moving belt-liquid chromatography–mass spectrometer interface.

Thin layer chromatography was performed on 0.25-mm silica gel plates (Mackerey-Nagel and Co.) containing material that fluoresces when exposed to UV light. The solvent system was 40:10:50 butanol–acetic acid–water, and chromatography proceeded for 3 h, during which time the solvent front traveled 120 mm. UV-absorbing compounds were detected as dark spots against the fluorescent background of the plate under UV light.

Purification and characterization of aldos-2-ulose dehydratase.—To prepare inoculum, *Polyporus obtusus* AU124PD (see below) was grown for 6 days in YM medium (Bacto Yeast Maltose broth) at 25° and 200 rpm. The culture was blended for 20 s in a sterile Eberbach blender, and 5 mL of the resulting suspension was used to inoculate each of 25 flasks containing 250 mL of YM medium. The cultures were grown for 8 days at 25° and 200 rpm, chilled, and filtered through a fine screen to harvest the mycelial balls. The mycelia were washed and resuspended in 2.5 L of 50 mM sodium phosphate, pH 7.0, containing 10 mM EDTA and 50 μg/mL of freshly solubilized phenylmethylsulfonyl fluoride (PMSF). This preparation was homogenized for 3 min using an Ultraturrax blender and then cleared by centrifuging at 10 K × *g* for 20 min.

Initial purification steps involved: (*1*) precipitation with 20% w/v PEG 4000 (60 min, 4°, centrifugation 15 min at 10 K × *g*, resuspension in the buffer used for blending, centrifugation 10 min at 10 K × *g*), and (*2*) removal of additional impurities from the supernatant by the addition of protamine sulfate to 0.2%, chilling to 4° for 15 min, and centrifugation at 10 K × *g* for 15 min. The resulting supernatant was purified using a DEAE-Sepharose column (5 × 16 cm, Pharmacia)

equilibrated with 30 mM Tris (pH 8.5, containing 2 mM EDTA and 10 μg/mL PMSF) and eluted with a gradient of 0–300 mM NaCl in the same buffer. The aldosulose-utilizing activity in the DEAE fractions was located using the A_{230} assay described above. Peak fractions were dialyzed, then rechromatographed on the same column. Peak fractions from the second DEAE-Sepharose column were dialyzed against 30 mM NaOAc, pH 5.5, and loaded onto a 2.5 × 18 cm column of carboxymethyl-Sepharose. The protein was eluted with a 0–200 mM gradient of NaCl in 50 mM NaOAc, pH 5.5. Protein concentration was measured by absorbance at 280 nm for column analysis and by the method of Lowry [13] (versus bovine serum albumin) for determination of specific activity. The enzyme was stable over a period of months at 4° in 0.1 M sodium phosphate buffer, pH 7.0.

RESULTS AND DISCUSSION

Evidence for the existence of an aldosulose-utilizing enzyme.—Partially purified pyranose 2-oxidase has been used to convert glucose into D-*arabino*-hexos-2-ulose, an intermediate in an enzymic process for the production of fructose in vitro [4]. Because this process for making D-*arabino*-hexos-2-ulose resulted in only a 90–95% yield, we examined whether the P2O preparation from *P. obtusus* might contain contaminating enzymes capable of generating byproducts from the aldosulose.

A number of reactions were run with P2O preparations of varying purity, using glucose as substrate. Fig. 1A shows the results of HPLC analysis of a reaction run at pH 6.0, utilizing 40% pure P2O. All of the glucose was consumed, and a large peak of the desired D-*arabino*-hexos-2-ulose product was seen. In addition, a large peak of early-eluting material was observed, apparently representing a byproduct. Formation of byproduct was dramatically reduced in a parallel reaction using highly purified P2O at pH 6.0 (Fig. 1B). The yield of D-*arabino*-hexos-2-ulose was also increased by using the more highly purified enzyme.

Additional experiments demonstrated that the 40% pure P2O preparation contained contaminants capable of acting specifically on the aldosulose, as opposed to glucose. Incubation of partially pure P2O with D-*arabino*-hexos-2-ulose resulted in conversion into the apparent byproducts (data not shown). Conversely, no D-*arabino*-hexos-2-ulose degradation products were detectable in incubations using a highly purified P2O preparation. In addition, the aldosulose did not appear to undergo significant chemical alteration during incubation at pH 6, even in the presence of proteins with whose amino groups it might conceivably react [15]. The product(s) of aldosulose consumption no longer reacted significantly with triphenyltetrazolium chloride (TTC) in a colorimetric assay for D-*arabino*-hexos-2-ulose [16]. Furthermore, it was found that a boiled preparation of partially pure P2O did not consume the aldosulose, indicating that consumption involved an enzymic, rather than a chemical, reaction.

Assays for the aldosulose-utilizing enzyme.—To facilitate purification and characterization of the aldosulose-utilizing enzyme, two sensitive and rapid spectrophoto-

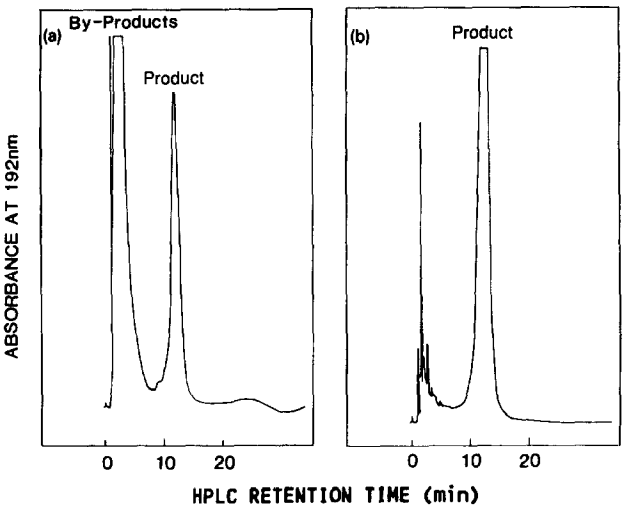

Fig. 1. HPLC analysis of enzymic reactions converting glucose to D-*arabino*-hexos-2-ulose and byproducts. Solutions of 2% D-glucose in 0.5 mL of 0.1 M sodium phosphate, pH 6.0, or 0.1 M sodium citrate, pH 4.4, containing 0.46 units of catalase, were treated with P2O preparations of varying purity and analyzed on a carbohydrate column as described in Materials and Methods. (a) 40% pure P2O, pH 6.0; (b) highly purified P2O, pH 6.0. In both cases, 0.46 units of P2O were added, sufficient to convert all of the glucose to the aldosulose in 2 h at 25° with 250 rpm shaking. Reactions were run a total of 6 h, and the column eluate was monitored at 192 nm in order to detect both the aldosulose (which absorbs below 200 nm) and byproducts.

metric assays were developed to measure the formation of its product. Under certain conditions the rate of increase of absorbance at either 230 or 265 nm was found to be a reliable assay for the enzyme (see Materials and Methods). To verify that these spectrophotometric assays were measuring an enzymic activity that was consuming D-*arabino*-hexos-2-ulose, the results were compared to those obtained when D-*arabino*-hexos-2-ulose consumption was measured directly using a TTC assay. When column fractions from the molecular-sieve chromatography of the aldosulose-utilizing enzyme were analyzed by all three assays, identical activity profiles (showing a peak at M_r 200 000) were obtained (data not shown). This indicated that the A_{265} and A_{230} assays correlated with the aldosulose-consuming activity and could be used in place of the more tedious TTC or HPLC assays.

pH Profile of the aldosulose-utilizing enzyme.—The pH optimum of partially purified aldosulose-utilizing enzyme was about 7.5 (Fig. 2). Activity at pH 4.5 was 15-fold lower than at pH 6.0. Since P2O is only slightly more active at pH 6.0 than at 4.5, this result appeared to explain the empirical observation that D-*arabino*-hexos-2-ulose made using impure P2O was produced at a much higher yield at pH 4.4 than at pH 6.0 (data not shown). Fewer byproducts were produced at pH 4.4 because of the reduced activity of the aldosulose-utilizing enzyme at that pH.

Testing the purity of the UV-absorbing product derived from D-arabino-*hexos-2-ulose.*—Tests were run on the 92% pure product obtained as described under Fig.

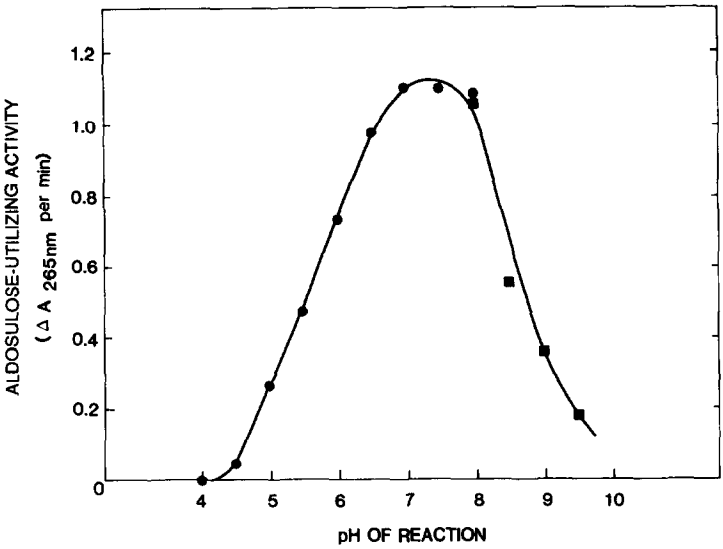

Fig. 2. pH Profile of the aldosulose-utilizing enzyme. Activity on 35 mM D-*arabino*-hexos-2-ulose was determined using the A_{265} assay described in Materials and Methods. ●, 50 mM citrate–50 mM phosphate buffer; ■, 100 mM Tris buffer.

3. The aldosulose substrate, the unknown product, and kojic acid (a related compound which may be derived biologically from D-*arabino*-hexos-2-ulose [17]) were analyzed by TLC as described in Materials and Methods. D-*arabino*-Hexos-2-ulose was not detectable by UV absorption, but when an equimolar amount of the unknown product was run, a single dark spot was observed, migrating slightly further than kojic acid. The UV spectrum of the product showed a single peak at 230 nm with molar absorbance of ~ 1500, indicative of the presence of an α,β-unsaturated ketone [18]. The combined analyses suggested that the UV-absorbing material produced by the aldosulose-utilizing enzyme was probably a single compound, possibly an α,β-unsaturated ketone.

Antibiotic activity of the aldosulose-derived product.—Studies reported by Baute and associates [1,2] showed that culture supernatants of the blue-pigmented fungus *Corticium caeruleum* contain an antibiotic that absorbs at 230 nm. The antibiotic, which they named cortalcerone, seemed to be made from glucose [1]. Working from the chemical structure they obtained for cortalcerone, they postulated that D-*arabino*-hexos-2-ulose might be an intermediate in its synthesis. In subsequent work, these authors succeeded in converting D-*arabino*-hexos-2-ulose to cortalcerone using unpurified mycelial macerates of *C. caeruleum* [3]. Consequently, we tested for antibiotic activity in the compound that had been synthesized from D-*arabino*-hexos-2-ulose in vitro using purified enzymes from *Polyporus obtusus*.

The D-*arabino*-hexos-2-ulose substrate did not inhibit growth of *E. coli*, when tested as described in Materials and Methods. However, a 6 mM solution of the product (derived enzymically from the same substrate preparation, as described

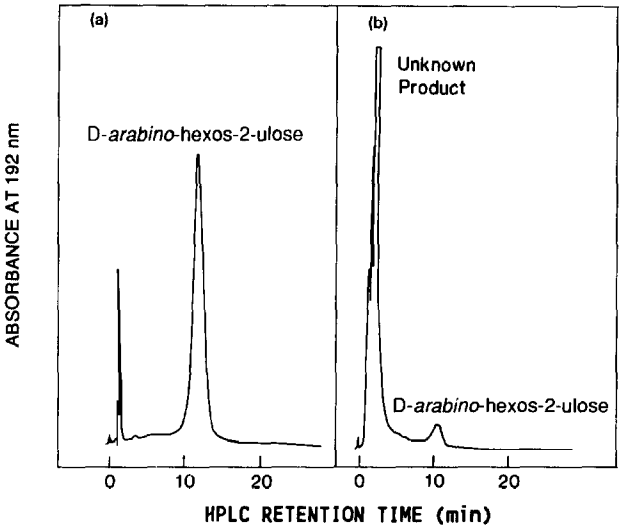

Fig. 3. Preparative synthesis of D-*arabino*-hexos-2-ulose and the product of the aldosulose-utilizing enzyme. Using 28 units of highly purified *P. obtusus* P2O and an equivalent amount of DEAE-Sepharose-purified *A. niger* catalase, 10 mL of a 6% solution of D-glucose in 0.1 mM sodium phosphate, pH 5.0, 1 mM in NaCl, was quantitatively converted into D-*arabino*-hexos-2-ulose in 3 h at 25° in a Radiometer pH-stat (TTT60 Titrator). No measurable acid or base was produced during the reaction. The protein was immediately removed by ultrafiltration through an Amicon PM30 filter, and the filtrate was analyzed by HPLC (panel a). To convert the D-*arabino*-hexos-2-ulose to the unknown product, the pH of the solution was adjusted to 6.0 with 1 mM NaOH, 3.5 mg of partially purified aldosulose-utilizing enzyme was added, and the pH of the solution was maintained at 6.0 in the pH-stat by addition of a total of 120 μL of 1 mM NaOH during the course of the reaction. Aliquots of the mixture were periodically monitored at 230 nm. After 80 min the rate of increase in absorbance at 230 nm had slowed, and a very small amount of acid production (presumably due to a side reaction) was beginning, so the reaction was terminated by chilling and rapid removal of the enzyme by PM30 ultrafiltration. An aliquot of the filtrate was analyzed (panel b), and the product was stored at −20°.

above) immediately inhibited all growth. The aldosulose-derived product also exhibited antibiotic activity against three other microorganisms when tested using a filter-paper assay on plates, as described in Materials and Methods. When combined with the spectral data, the results supported the hypothesis that the unknown substance was cortalcerone, or a compound closely related to it.

Spectral analysis of the aldosulose-derived product shows it to be cortalcerone.— The deproteinized product derived from D-*arabino*-hexos-2-ulose was characterized by ^{13}C- and ^{1}H-NMR, IR, and mass spectrometry. The ^{13}C-NMR spectrum in D_2O showed signals indicating six different carbons in the unknown product. These appeared at 194, 150, 125, 95, 90, and 61 ppm. The ^{1}H-NMR spectrum in D_2O had signals at 7.8 (1 H, multiplet), 6.6 (1 H, multiplet), 5.7 (1 H, singlet), and 5.05 ppm (2 H, multiplet). These results are consistent with the reported structure of cortalcerone [19], containing α,β-unsaturated ketone, hemiacetal, and hydrated aldehyde functions.

The IR spectrum confirmed the presence of hydroxyl groups and a conjugated carbonyl, showing peaks at 3400, 1695, 1630, 1470, 1440, 1370, 1240, 1080, 1030, 990, 950, 920, and 810 cm^{-1}. The chemical ionization mass spectrum indicated a molecular weight of 160 for the product. The major peaks (relative intensity, %) in the spectrum were at m/z 161 (42) $[M + H]^+$, 143 (100) $[160 - H_2O]$, 125 (85) $[143 - H_2O]$, 115 (48) [143 – CO], 99 (85), and 97 (54) [125 – CO].

Our spectral data are consistent with those previously obtained by Baute and coworkers for cortalcerone isolated from macerates of *C. caeruleum* [2,19]. The differences in mass-spectral fragmentation are the result of differences in ionization methods. Thus, we believe that our unknown product is the same compound. The structure of cortalcerone has been determined by previous researchers, using X-ray crystallographic analysis [20].

Reaction intermediates in the biosynthesis of cortalcerone.—As described above, significant UV absorbance changes occur during the conversion of D-*arabino*-hexos-2-ulose into cortalcerone. The following kinetic experiments demonstrate that these spectral changes reflect the chemical properties of intermediates associated with the biosynthesis of cortalcerone, and of cortalcerone itself.

Since the aldosulose-utilizing enzyme was shown to have a pH optimum of 7.0–8.0 (Fig. 2), initial spectral assays during cortalcerone synthesis were performed at pH 7.0. However, it is known that D-*threo*-pentos-2-ulose, the 5-carbon analogue of D-*arabino*-hexos-2-ulose, is not stable at alkaline pH [21]. In fact, in the absence of added enzyme we observed a slow breakdown of D-*arabino*-hexos-2-ulose, at pH 7.0, to a compound absorbing maximally at 310 nm. When the same reaction was performed at pH 6.0 or lower, chemical breakdown of D-*arabino*-hexos-2-ulose (as measured by absorbance increase at 310 nm) was insignificant. Consequently, all subsequent reactions were done at pH 6.0.

When the aldosulose-utilizing enzyme was added to D-*arabino*-hexos-2-ulose at pH 6.0, a compound absorbing at 265 nm accumulated rapidly, reaching a plateau in a matter of minutes (Fig. 4a). The plateau concentration of this material was proportional to the amount of enzyme added, as expected of a steady-state reaction intermediate (data not shown). Following a brief lag, the concentration of cortalcerone (absorbing at 230 nm) increased at a constant rate. When the reaction mixture was rapidly deproteinized by ultrafiltration, further changes leading to chemical equilibrium could be followed by changes in the spectrum (Fig. 4b). The steady-state intermediate absorbing at 265 nm was seen to decrease rapidly, probably as a result of chemical rearrangement to another compound lacking UV absorbance, but the cortalcerone peak at 230 nm did not increase. Thus, it appeared that the steady-state intermediate was being transformed into another, nonabsorbing intermediate that required an additional enzyme-catalyzed step to form cortalcerone. The data shown in Fig. 4c supported this hypothesis. When enzyme was added back to the reaction mixture, cortalcerone synthesis resumed at essentially the maximal rate without a lag, while the steady-state intermediate that absorbed at 265 nm was eventually replenished from the remaining aldosulose

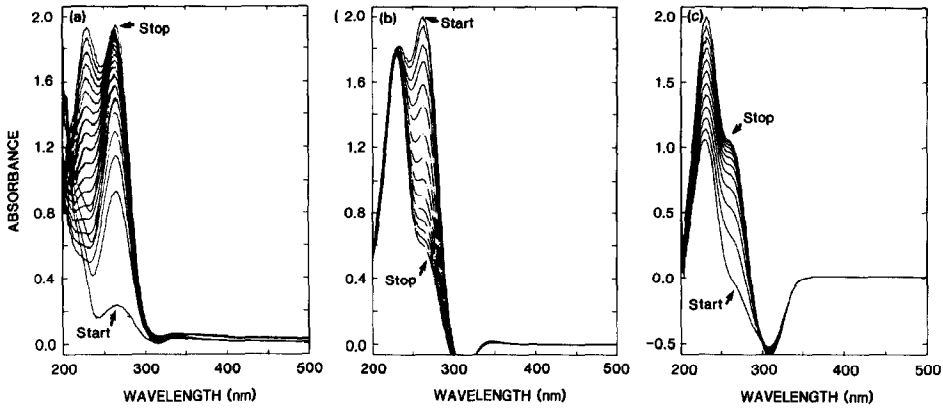

Fig. 4. Spectral changes observed during the in vitro synthesis of cortalcerone from D-*arabino*-hexos-2-ulose. Panel (a): a 0.5% solution of pure D-*arabino*-hexos-2-ulose in 1 mL of 0.1 M sodium phosphate buffer, pH 6.0 was incubated with ~12 μg of partially purified aldosulose-utilizing enzyme. Spectra were recorded every 30 s using a Hewlett–Packard 8450A spectrophotometer. Panel (b): after the reaction mixture had been incubated for 11 min, it was deproteinized by ultrafiltration (in 90 s) and re-examined for spectral changes every 30 s (solid lines) or 85 s (broken lines). Panel (c): after the deproteinized solution had been incubated for 16 min, aldosulose-utilizing enzyme was added back to the original concentration, and spectral changes were monitored every 30 s. (The decrease in absorbance at 310 nm apparently reflects removal of material during the ultrafiltration step, since the absorbance at 310 nm did not change during the course of the reaction with or without enzyme.)

substrate. Thus, the aldosulose-utilizing enzyme appeared also to act on a second substrate — the nonabsorbing compound produced by chemical transformation of the steady-state intermediate.

A biosynthetic pathway from glucose to cortalcerone.—Based on the kinetic and spectral data it was possible to draw a multistep pathway for the biosynthesis of cortalcerone that appeared to explain all of the experimental observations (Fig. 5). The pathway probably represents what occurred in the in vitro reactions, and we propose that the same reactions also occur under certain physiological conditions in the fungal cell.

The P2O-catalyzed conversion of glucose into D-*arabino*-hexos-2-ulose has previously been partially characterized [11]. We propose that the product of the P2O reaction is a specific tautomeric form of D-*arabino*-hexos-2-ulose (form IV). This form has been shown by Hindsgaul (personal communication and also reported by Shaked and Wolfe [22]) to be in equilibrium with three other forms of D-*arabino*-hexos-2-ulose in solution (Fig. 5). One of these forms (II or IV) is apparently a substrate for the aldosulose-utilizing enzyme, which dehydrates D-*arabino*-hexos-2-ulose, generating a double bond between C-3 and C-4. This unsaturated compound would be expected to absorb in the UV and is proposed to be the steady-state intermediate characterized by the spectral peak at 265 nm (Fig. 4a). This intermediate was shown to be chemically unstable (Fig. 4b), and it might be expected to rearrange from its strained 1,5 ring form to a more stable 2,6 pyranose structure. As this intermediate no longer has conjugated unsaturation, its proposed

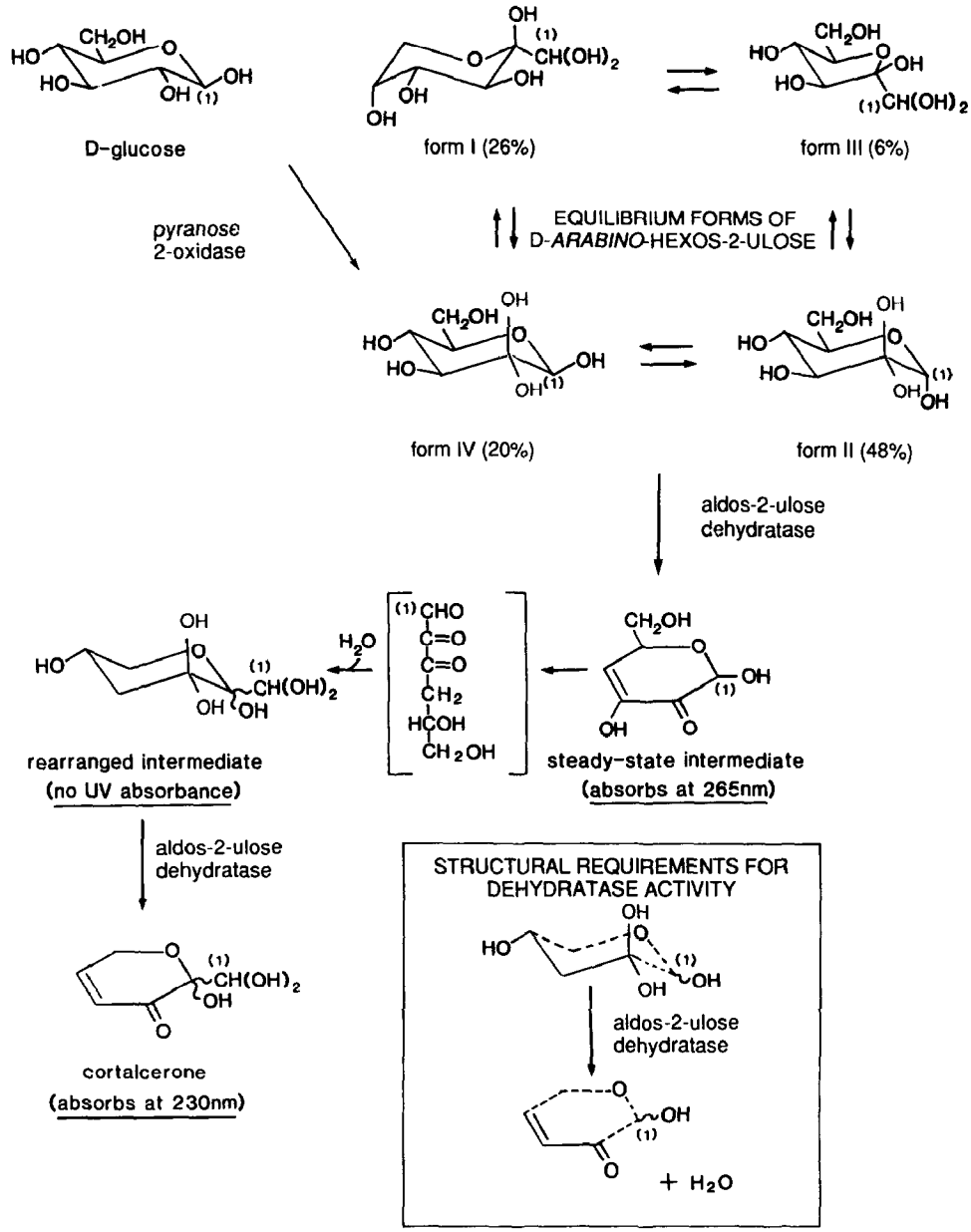

Fig. 5. Proposed pathway for the biosynthesis of cortalcerone in *P. obtusus*. The scheme shows the enzymically catalyzed steps, as well as the chemical rearrangements, that appear to occur during the conversion of glucose into cortalcerone in vitro. Carbon 1 in each molecule is indicated.

structure agrees with the observed loss of UV absorbance at this stage of the synthesis (Fig. 4b).

At this point in the proposed pathway, structure and configuration at C-3, -4, and -5 of the rearranged intermediate resemble those at C-2, -3, and -4 of the

original aldosulose substrate. Unexpectedly, the aldosulose-utilizing enzyme appears to act a second time, dehydrating this pyranose structure to form cortalcerone. Thus, the structural requirements for enzymic activity can apparently be generalized as shown in the inset in Fig. 5. Based on the conserved features of the substrates, we propose that the aldosulose-utilizing enzyme be referred to as aldos-2-ulose dehydratase.

Testing the proposed biosynthetic pathway by examining the reactions of D-threo-*pentos-2-ulose.*—If the model shown in Fig. 5 is correct and the structural requirements for enzyme activity are as shown in the inset, an interesting result is predicted for the reaction of the enzyme with D-*threo*-pentos-2-ulose (also known as D-xylosone). When xylose is converted into D-*threo*-pentos-2-ulose using P2O, the product is presumably identical to D-*arabino*-hexos-2-ulose at C-2, -3, and -4, but lacks a 6th carbon (Fig. 6). Consequently, it was predicted that the aldosulose-utilizing enzyme, aldos-2-ulose dehydratase, should also dehydrate D-*threo*-pentos-2-ulose to produce a compound absorbing near 265 nm. Because of its strained nature, the intermediate might still rearrange, losing UV absorbance, but since it *lacks* the 6th carbon, it could not form a 2,6 pyranose ring to generate a second substrate for the enzyme. Thus, in a reaction using D-*threo*-pentos-2-ulose, a product absorbing near 265 nm might be formed, but no conversion into a 230 nm-absorbing product would occur. This is the result that was obtained (Fig. 6, inset), apparently confirming the proposed model. Because the enzyme is not active on related compounds such as fructose (data not shown), yet does dehydrate three different aldos-2-uloses, the name aldos-2-ulose dehydratase seems appropriate.

Purification and characterization of aldos-2-ulose dehydratase.—During the course of our experiments, a spontaneous variant of *P. obtusus* strain AU124 was isolated that produced up to 40-fold more aldos-2-ulose dehydratase activity. This strain (AU124PD), whose P2O production was unaffected, was used to generate an aldos-2-ulose dehydratase preparation that was purified 450-fold (Table I).

Based on kinetic analysis of the data shown in Figs. 4a–c, it appeared that aldos-2-ulose dehydratase catalyzes both of the dehydrations required to produce cortalcerone. An alternative explanation (that one of the two steps is catalyzed by a *different* enzyme present in the partially pure preparation) was also examined. When the catalytic properties of the purified enzyme preparation were compared with those of the crude lysate, both of the proposed dehydration activities were observed in the same ratio (Table I). Since the two catalytic activities are apparently not separable, we tentatively conclude that aldos-2-ulose dehydratase does catalyze both of the steps attributed to it in the pathway proposed in Fig. 5.

The apparent native MW of aldos-2-ulose dehydratase is approximately 200 000, as determined by Bio-Gel P-300 molecular sieve chromatography (Fig. 7). When equal aliquots of selected fractions of the sizing column were analyzed by SDS-PAGE, a predominant band was seen at approximately 95 kDa (Fig. 7, inset). The intensity of the 95 kDa band paralleled the amount of enzyme activity seen in the same fractions, indicating that it probably represents the enzyme subunit. Thus,

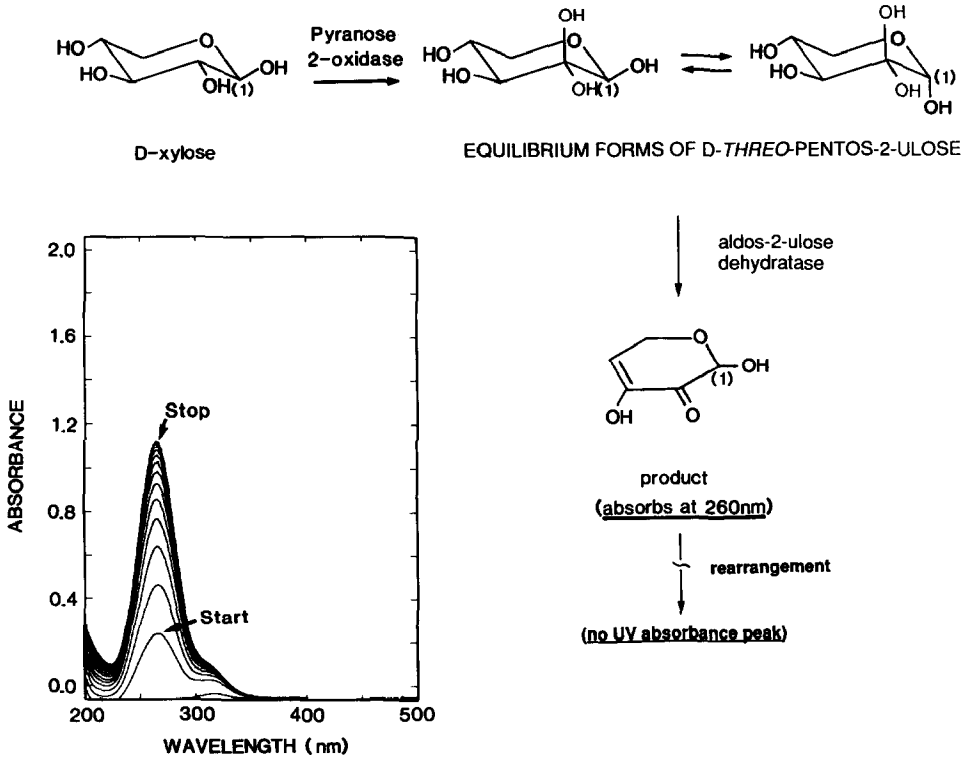

Fig. 6. Activity of aldos-2-ulose dehydratase on D-*threo*-pentos-2-ulose. The predicted course of the reaction is shown in the formula sequence. Carbon 1 in each molecule is indicated, and the structures shown for D-*threo*-pentos-2-ulose are the two major forms known to be present in solution [23]. To prepare D-*threo*-pentos-2-ulose, a 2% solution of D-xylose in 0.1 M sodium phosphate buffer, pH 6.0, was incubated with *P. obtusus* P2O and *A. niger* catalase for 4 h at 25°. The solution was deproteinized by Amicon PM30 ultrafiltration, and a 0.25% solution of the product was used as a substrate for the same aldosulose dehydratase used to obtain the data in Fig. 4. Spectra taken every 3 min are shown in the inset.

aldos-2-ulose dehydratase appears to be a dimer of ~95 kDa subunits. If the subunits are identical, there may be only one type of active site per subunit, and that site may be responsible for both of the proposed dehydrations shown in Fig. 5.

CONCLUSIONS

We have characterized a synthetic pathway in which D-glucose is converted, via D-*arabino*-hexos-2-ulose, into the antibiotic cortalcerone (5,6-dihydro-6-hydroxy-5-oxo-2*H*-pyran-6-carboxaldehyde hydrate). The antibiotic has been produced in vitro in essentially pure form using two purified enzymes from the wood-rotting fungus, *Polyporus obtusus*, and the reactions can be driven to near completion at

TABLE I

Purification of aldos-2-ulose dehydratase

Preparative step	Total protein (mg)	Volume (mL)	Total units by 230 nm assay [a]	Specific activity (U/mg protein)	Fold purification	Recovery (%)	Activity ratio [b] (A_{265}/A_{230})
Cleared lysate	3880	3530	77.6	0.02	1	100	18
PEG precipitate	825	150	34.3	0.07	2	47	21
1st DEAE-Sepharose column	143	140	29.5	0.21	10	38	19
2nd DEAE-Sepharose column	53	52	20.8	0.40	20	26	23
CM-Sepharose column	0.83	13.5	7.5	9.0	450	9.6	24

[a] See Materials and Methods. [b] Preparations were first assayed by the A_{265} procedure (Materials and Methods). Portions containing equivalent numbers of A_{265} units were reassayed at 25°, pH 6.0, using 99.9% pure D-*arabino*-hexos-2-ulose at a final concentration of 125 mM. Approximate "initial rates" of reaction were determined (*1*) within the first minute (for the rate of formation of product absorbing at 265 nm), and (*2*) after 15 min (for the rate of formation of product absorbing at 230 nm).

relatively high substrate concentrations. The final product of this enzymic conversion is significantly purer than the products of most chemical reactions involving D-*arabino*-hexos-2-ulose [6]. Thus, the enzymic synthesis of cortalcerone in vitro overcomes the problems associated with its synthesis by chemical methods [25]. The aldosulose-utilizing enzyme, which also acts on D-*threo*-pentos-2-ulose, may be of use in the efficient production of a variety of compounds derived from other osuloses in vitro.

Similarly, it is now very easy to produce concentrated solutions of D-*arabino*-hexos-2-ulose or D-*threo*-pentos-2-ulose of over 99% purity by using pyranose 2-oxidase that is free of aldosulose-utilizing enzyme. The fact that the gene for P2O has been cloned [26] could facilitate production of D-*arabino*-hexos-2-ulose on a larger scale through the use of the recombinant enzyme.

We have presented a model that shows the enzymic and chemical steps apparently involved in the synthesis of cortalcerone from glucose in vitro (Fig. 5). This biosynthetic pathway includes four separate steps. Experimentally we find evidence for four steps, although they seem to be accomplished in vitro through the use of just two enzymes. Our explanation for this phenomenon is that the oxidative step is catalyzed by P2O, one step is a spontaneous chemical rearrangement, and *both* of the dehydrations are catalyzed by one enzyme, which we have designated aldos-2-ulose dehydratase. If, as evidence suggests, this enzyme is a homodimer of 95 kDa subunits, the two chemically related dehydrations may occur at a single type of active site.

The existence of an aldosulose-dehydrating enzyme was originally postulated by Baute [1], who subsequently suggested several possible pathways for the conversion of D-*arabino*-hexos-2-ulose into cortalcerone in crude extracts of *Corticium*

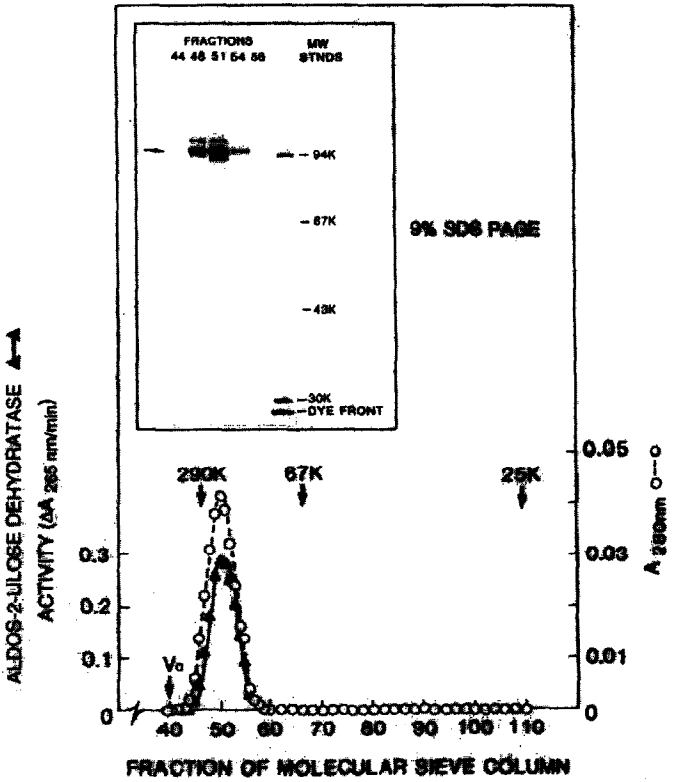

Fig. 7. Apparent native and subunit molecular weights of purified aldos-2-ulose dehydratase. The native enzyme, purified as summarized in Table I, was passed through a 1.5×95 cm Bio-Gel P-300 molecular sieve column in 0.5 M sodium phosphate, pH 7.0, and its elution volume was compared with those of catalase, bovine serum albumin, and chymotrypsinogen standards. Fractions of the activity peak were concentration by TCA precipitation, rinsed in acetone–ethanol, boiled in reducing SDS buffer, and subjected to 9% SDS-PAGE [24]. Proteins were visualized by staining with Coomassie Brillant Blue (see inset).

caeruleum. The pathway involving a bicyclic form of D-*arabino*-hexos-2-ulose (Baute et al. [27]) is not shown in our scheme because there is no evidence that significant amounts of this form ever occur in solution. Furthermore, it is not clear that a dehydration is required between compounds **4** and **5** in this pathway (Fig. 1 of ref. 27). While it is possible that biosynthetic pathways such as those suggested by Baute et al. do exist in *C. caeruleum*, no characterization of their proposed dehydrating enzyme has yet been published. Thus, the alternative pathway described here and proposed earlier for cortalcerone synthesis in *P. obtusus* [28] may also exist in *C. caeruleum* and other fungi in which cortalcerone production has been detected.

The end-product formed from D-*arabino*-hexos-2-ulose by aldos-2-ulose dehydratase in vitro was shown to have antibiotic activity. Consequently, one biological function of pyranose 2-oxidase appears to be the production of D-*arabino*-hexos-2-

ulose as a precursor of cortalcerone. Both pyranose 2-oxidase and aldos-2-ulose dehydratase appear to be induced late in the growth cycle of *P. obtusus*, as glucose concentration becomes limiting (Koths and Halenbeck, unpublished observations). Thus cortalcerone, like other fungal secondary metabolites having antibiotic activity, may be valuable to the organism during periods of slow growth. Cortalcerone (or compounds derived from it) might also serve to facilitate sporulation by inhibiting germination, as has been proposed for trichodermin, another fungal metabolite with antibiotic activity [29]. In this regard, it is interesting to note that many compounds containing the dihydro-2*H*-pyran-5-one structure of cortalcerone have been shown to have antibacterial and antifungal activities at low concentrations [30]. The production of such compounds by other organisms also suggests that cortalcerone may be further modified by organisms that produce it.

D-*arabino*-Hexos-2-ulose is known to be produced by cultures of various fungi, as well as by the mold *Aspergillus flavus*, in which its biosynthesis was originally observed [31]. Baute and Baute [32] have reported that a significant proportion of 315 macrofungal species tested appear to produce D-*arabino*-hexos-2-ulose from glucose. Forty-three of these fungi also were able to make a product having the chemical properties expected of cortalcerone. Hence, cortalcerone and its biosynthetic pathway appear to have a relatively widespread significance, at least in macrofungal systems. *Coriolus versicolor* and *Lenzites betulinus* are examples of white-rot fungi that we have shown to produce D-*arabino*-hexos-2-ulose from glucose, using pyranose 2-oxidases [33]. Preliminary experiments suggest that these fungi do not contain an aldosulose-utilizing enzyme of the type described above and do not produce cortalcerone. Consequently, D-*arabino*-hexos-2-ulose, as an end-product, may have a biological role in certain fungal systems.

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