Journal of Natural Products
Vol. 47, No. 4, pp. 692-697, Jul-Aug 1984

OXIDATION OF ROTENONE BY *POLYPORUS ANCEPS* LACCASE

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ABSTRACT.—The extracellular laccase produced by *Polyporus anceps* transforms rotenone to a single, more polar product. This transformation occurs in incubation mixtures containing chlorpromazine, laccase, and rotenone where rotenone serves as a pseudosubstrate for the enzyme. Chlorpromazine, the true substrate, serves as a cycling redox component of the system forming a radical-cation species that abstracts an electron from rotenone in the oxidation process. Physicochemical properties of the product were determined on an analytically pure sample obtained by preparative hplc. High resolution ms, high-field pmr and cmr, uv, and optical rotation analyses indicated that rotenone had been transformed to 6aβ, 12aβ-rotenolone by *P. anceps* laccase.

The natural product rotenone (**1**) is a widely used pesticide. The compound possesses a range of biological activities, including its ability to induce neoplastic, paraneoplastic, and preneoplastic lesions in rats (1, 2). Rotenone is susceptible to microbial (3) and photochemical oxidation (4). The enzymes of microorganisms catalyze the introduction of molecular oxygen into the isopropylene side-chain of rotenone, while photohydroxylation accomplished in air gives rise to 6aβ, 12aβ-rotenolone (**2**) as the major product. Other rotenolone isomers are apparently unstable and form as transient intermediates leading to the more stable *cis*-rotenolone isomers like **2**. In general, products formed by photochemical oxidation of rotenone were less toxic than rotenone when administered to mice.

Laccases (E.C. 1.14.18.1) are commonly found among members of the Ascomycetes, Basidiomycetes, and the Fungi Imperfecti. These enzymes are copper-containing proteins that utilize molecular oxygen as the terminal electron acceptor (5-7). The extracellular laccases of *Polyporus* and *Rhizoctonia* species transform natural products like vindoline (8) and phenolic substrates (5). In general, reactions catalyzed by laccases involve free radical intermediates similar to those implicated in photochemical oxidations. This similarity to the proposed pathways of photochemical oxidation of rotenone prompted an investigation of the nature of the metabolite produced by the action of the laccase from *Polyporus anceps* Peck on rotenone. This report describes the production, isolation, and identification of 6aβ, 12aβ-rotenolone (**2**) as the major product formed by the oxidation of rotenone by laccase.

EXPERIMENTAL

ENZYME PRODUCTION.—*P. anceps* was grown according to the previously described procedure (9). The laccase produced in the culture supernatants was bound to DEAE-cellulose, H^+, medium mesh (No. D8382, Sigma) and treated as described before (9).

ENZYME ASSAY.—Laccase was assayed as described before (9) and protein concentration determined by the method of Bradford (10).

INCUBATION OF LACCASE WITH ROTENONE (**1**) AND CHLORPROMAZINE.—Moist DEAE-bound laccase (136 g) was suspended in 975 ml of pH 5.0, 0.2M potassium phosphate solution, and the solution was filtered through a glass column plugged with glass wool. The clear light yellow filtrate contained 7.4×10^{-2} unit ml^{-1} of laccase activity and 50 μg ml^{-1} of protein. Small scale incubations were carried out in DeLong flasks (125 ml capacity) containing 10 ml (0.74 units) of laccase solution. Rotenone (5 mg, dissolved in 0.1 ml $CHCl_3$) and chlorpromazine (5 mg) were added to the flasks, which were shaken at 200 rpm at 28° for 4 h. The reaction mixtures were then extracted three times with ½ volume of EtOAc. Tlc of the extract on silica gel GF 254 (Merck) in C_6H_6-MeOH (49:1), indicated the presence of a new compound

(Rf 0.26) in the mixture along with unreacted rotenone (Rf 0.45). Control incubations consisted of mixtures of laccase and rotenone (**1**) without chlorpromazine; laccase and chlorpromazine but no rotenone; and rotenone plus chlorpromazine but no laccase.

PREPARATIVE-SCALE PRODUCTION OF THE ROTENONE OXIDATION PRODUCT.—Incubations were carried out in nine 1-liter DeLong flasks, each containing 7.4 units of laccase in 100 ml of solution. Rotenone (580 mg, dissolved in 0.9 ml $CHCl_3$) and chlorpromazine (580 mg) were dispersed evenly among the flasks. The incubation period and the extraction procedure used were identical to those employed for small-scale experiments.

ISOLATION OF THE LACCASE PRODUCT.—The crude concentrated EtOAc extract (760 mg) was applied to a silica gel column (85g, 60-200 mesh, Baker Analyzed Reagents #3404), which had been slurry-packed in the developing solvent of C_6H_6-MeOH (20:1). Fractions (11 ml) were collected in a Fractomette 200 instrument, and chromatographically similar fractions (*18-30*) were combined and concentrated to dryness under vacuum. The residue obtained (631 mg) was further purified by preparative tlc (0.5 mm layer) using C_6H_6-MeOH (49:1) as the developing solvent. The band centered at Rf 0.26 containing the laccase oxidation product was scraped off the plates and exhaustively eluted with EtOAc. The eluant was dried under vacuum to give 55 mg of the chromatographically pure product as a noncrystalline solid. Further purification of this material was achieved by preparative hplc. Samples dissolved in MeOH were analyzed by hplc using MeOH-H_2O (75:15) as the solvent. The analytically pure sample (24 mg) was obtained by collecting the peaks centered at an elution volume of 41.26 ml. This sample was used for structure elucidation.

INSTRUMENTATION.—Uv/visible spectra were obtained using a Hewlett-Packard 8450A UV/visible spectrophotometer connected to a Hewlett-Packard 7470 plotter. High-resolution mass spectra were taken on a Kratos AEI MS-50 instrument through the mass spectra services of the Department of Chemistry, University of Nebraska, Lincoln, Nebraska. Pmr and cmr spectra were obtained at 500 MHz on a Bruker WM-500 or a Bruker WM-360 FTQNMR spectrometer, using $CDCl_3$ as solvent with TMS as internal standard. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. Hplc was conducted using a Waters Associates instrument (ALC/GPC 202) and a uv detector at 280 nm with a semi-preparative Partisil 10 ODS M9 C18-column (Whatman magnum 9) particle size 10 μm (500×9.4 mm). The operating pressure was 1300 psi. The samples were injected through a Waters Universal injector.

MATERIALS.—Rotenone (**1**) was purchased from Sigma Chemical Co., St. Louis, Missouri, and possessed physical properties (mp, pmr and cmr, optical rotation, mass spectrum) identical to those reported in the literature (11-14). Syringaldazine was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, and chlorpromazine was purchased from Sigma Chemical Co.

RESULTS AND DISCUSSION

Small-scale incubation experiments demonstrated that laccase oxidized rotenone (**1**) to a single, more polar product in the presence of chlorpromazine. The extent of conversion of (**1**) in these incubations was estimated to be 20% in 4 h, and longer incubation periods did not improve the extent of conversion. Incubations of laccase lacking chlorpromazine or controls containing buffer and chlorpromazine and rotenone and buffer did not result in the production of the polar product.

PROPERTIES OF THE SUBSTRATE.—Rotenone (**1**) indicated the following physical properties: mp, 160°; λ max (MeOH) 293 (log ϵ 4.2), 242 (log ϵ 4.1) and 235 nm (log ϵ 4.1); optical rotation $[-100]^{28}D$, 0.1 g/100 ml ($CHCl_3$); hrms *m/z* (% relative abundance) 394.1417 (100, calc for $C_{23}H_{22}O_6$ 394.1416), 192 (60), 191 (18), 177 (10); pmr data, see discussion and inset in Figure 1; cmr data, see Table 1.

PROPERTIES OF THE LACCASE-OXIDATION PRODUCT.—The laccase metabolite of rotenone gave physical properties which were compared directly with rotenone: optical rotation $[-145]^{28}D$, 0.1 g/100 ml ($CHCl_3$); hrms *m/z* (% relative abundance) 410.1361 (20.5, calc for $C_{23}H_{22}O_7$ 410.1608), 392 (24.96), 208 (100), 192 (5); pmr data see discussion and Figure 1; cmr data see Table 1.

A wide range of natural and synthetic products are oxidized by copper oxidases including laccases and ceruloplasmin (5, 8). Free radical intermediates are implicated in copper oxidase oxidation reactions and some substrates interact directly with these en-

zymes. Other substrates are only indirectly oxidized by these enzymes and require the intermediacy of "cofactors" in the oxidation process. In such oxidative pathways, compounds like hydroquinone, catechol, DOPA, and chlorpromazine are the true substrates for the enzyme, while compounds such as rotenone are indirect or "pseudosubstrates" for the copper oxidases. In our earlier work, chlorpromazine served as a convenient and excellent cofactor in laccase and human serum ceruloplasmin oxidation reactions, and it was selected for the present work with rotenone. The role of cofactors in the oxidation of rotenone by laccase is illustrated in Figure 2. Molecular oxygen serves as the terminal electron acceptor in the oxidation process where rotenone most likely loses an electron and a proton from position-6a. In oxidations with alkaloids, laccase converts chlorpromazine to a radical-cation intermediate as a first step. In our studies with rotenone, laccase oxidations do not occur in the absence of cofactor.

Following a preparative scale incubation, the analytically pure sample of the metabolite was obtained by preparative hplc. Proof of the structure of the laccase oxidation product relied on a combination of spectral and physical analyses. The mass spectral fragmentation patterns of rotenoids are well characterized (12). The primary fragmentation results in cleavage of the molecule into ions containing elements of ring A and B (*m/z* 192 for rotenone). The molecular ion in the high-resolution ms of the metabolite gave *m/z* 410.1361 for $C_{23}H_{22}O_7$, indicating the presence of a single additional oxygen atom vs rotenone ($C_{23}H_{22}O_6$) in the structure of the product. The base peak of the spectrum occurred at *m/z* 208 with an empirical formula of $C_{11}H_{12}O_4$, indicating that the location of the additional oxygen atom was either on ring A or B of the product structure.

Chemical shift assignments have been made for most proton signals in the nmr spectrum of rotenone (3, 13-15). Aromatic protons H-7 and H-10 of ring A appear as two sharp singlets at 6.77 and 6.44 ppm, respectively. The signal for H-7 serves as a valuable indicator of structural changes at positions 6 and 6a, and of the geometry of the B/C ring fusion. A *trans*-B/C ring juncture subjects H-7 to the deshielding effects of the carbonyl functional group, moving this signal to about 8 ppm. Proton signals for H-4 and H-5 occur as a characteristic AB-pattern centered at 6.49 and 7.83 ppm ($J=11$ Hz), respectively. The signal for H-12a is obscurred in low field pmr spectra, and that for H-6a is coincidental with a methoxyl group signal at 3.79 ppm. Unambiguous assignments of these other signals in the metabolite and rotenone spectra were made by use of 360 and 500 MHz nmr.

The 360 MHz spectrum of rotenone (**1**) is shown as an inset in Figure 1. In this spectrum, the signal for H-6a is clearly seen as a doublet at 3.83 ppm ($J=4.63$ Hz). The broad singlet at 4.92 ppm integrates for two protons, one of which has been assigned to H-12a by Unai *et al.*, (15), the other to an olefinic signal from H-1′.

The high-field nmr spectra of the laccase oxidation product were nearly identical to rotenone for most resonances. However, in the metabolite spectrum, the signal for H-6a was absent and the signal pattern for aromatic protons of ring-A was different. The H-10 proton signal still resonated at 6.48 ppm, but that for H-7 was shifted upfield by 0.23 ppm to 6.54 ppm. In addition, one of the two protons in the singlet at 4.9 ppm was shifted to 4.58 ppm in the metabolite spectrum. In the metabolite, signals for H-12a and one of the H-12 protons overlap at 4.58 ppm and integrate for 2 protons (Figure 1). These pmr spectral observations are similar to those made by Unai *et al.* (15) for the structure of 6aβ, 12aβ-rotenolone (**2**) and strongly suggest that the metabolite structure is **2**.

Cmr spectral analyses have enabled the complete assignment of signals for all of the carbon atoms of rotenoids except for rotenolones like **2** (11). Nearly all of the signals in the cmr spectrum of the laccase product were identical to those for rotenone except for

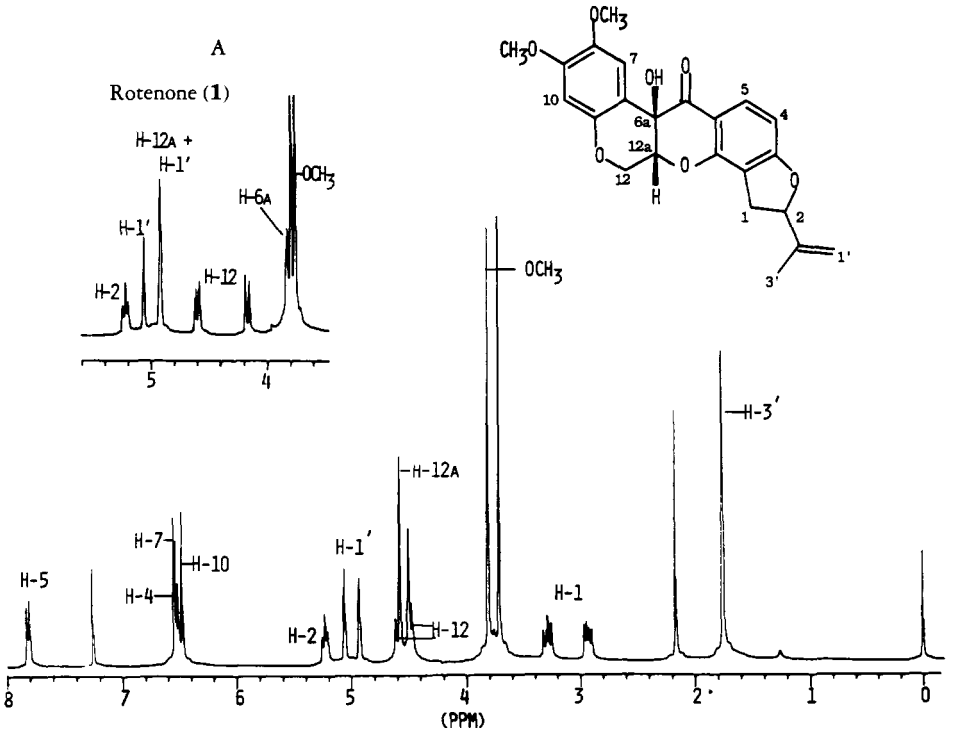

FIGURE 1. Pmr spectrum of rotenolone (**2**) at 360 MHz together with the expanded regions. The inset A, includes pertinent regions of the pmr spectrum of rotenone (**1**).

C-6a and adjacent carbons (Table 1). The signal for C-6a in the metabolite occurs at 67.57 ppm as a singlet nearly 22.97 ppm downfield from the doublet carbon signal observed in rotenone. The magnitude of the shift, the formation of a new singlet signal for C-6a, and the deshielding effects of a new hydroxyl group at position C-6a on adjacent carbon atoms are all consistent for the metabolite structure as **2**.

The stereochemical features of rotenolone epimers have been determined by optical rotation measurements (15). The value reported for the optical rotation of the *cis*-fused 6aβ, 12aβ-rotenolone (**2**) is −190° (15), while the isomeric *trans*-fused 6aα, 12aα-rotenolone had a rotation of +31°. The optical rotation of the laccase product after repeated purification through preparative hplc was −145°. This strongly negative value, while different from that reported by Unai *et al.* (15), supports the *cis* B/C ring junction with a 6a-orientation of the hydroxyl group.

The laccase oxidation product has been fully identified as the *cis*-fused, 6aβ, 12aβ-rotenolone (**2**). This compound is also formed by photochemical oxidation, and it is an expected photodecomposition product from crops treated with rotenone as a pesticide (4). It is interesting that the mechanism of laccase oxidation of other substrates using an identical enzyme incubation mixture involves a radical-cation intermediate that forms when chlorpromazine is oxidized by the copper oxidase (8). In this way, the laccase oxidation process represents a mimic of the photochemical oxidation of rotenone.

The rotenolone (**2**) possesses similar biological and toxicological properties to rotenone (4). These compounds both inhibit the oxidation of NADH in mitochondria at the level of NADH-dehydrogenase (16, 17). Metabolites like **2**, which retain the E-ring intact, have inhibitory activities comparable to rotenone (18). The widespread occurrence of laccases in nature (i.e., plants, fungi, and in bacteria) (6), together with the fact that rotenone is widely used as a non-persistent pesticide, indicates the high probability that rotenone is oxidized by these and perhaps other enzymes in nature.

TABLE 1. Cmr Assignments for Rotenone (**1**) and the Oxidation Product (**2**)

Carbon	Chemical shift (ppm)[a]	
	rotenone **1**	metabolite **2**
1	31.3t	31.17t
2	87.8d	87.94d
3a	167.4s	167.96s
4	104.7d	105.26d
5	130.0d	130.03d
5a	114.7s	111.75s
6	188.9s	191.01s
6a	44.6d	67.57s
6b	104.7s	108.82s
7	110.4d	109.59d
8	143.9s	144.02s
9	149.5s	151.18s
10	100.9d	101.12d
10a	147.4s	148.40s
12	66.3t	63.85t
12a	72.2d	76.08d
13a	156.1s	157.63s
13b	113.0s	113.15s
1′	112.6t	112.6t
2′	143.0s	142.86s
3′	17.2q	17.09q
OMe	55.8q	55.85q
OMe	56.3q	56.43q

[a]The spectra were obtained in $CDCl_3$.

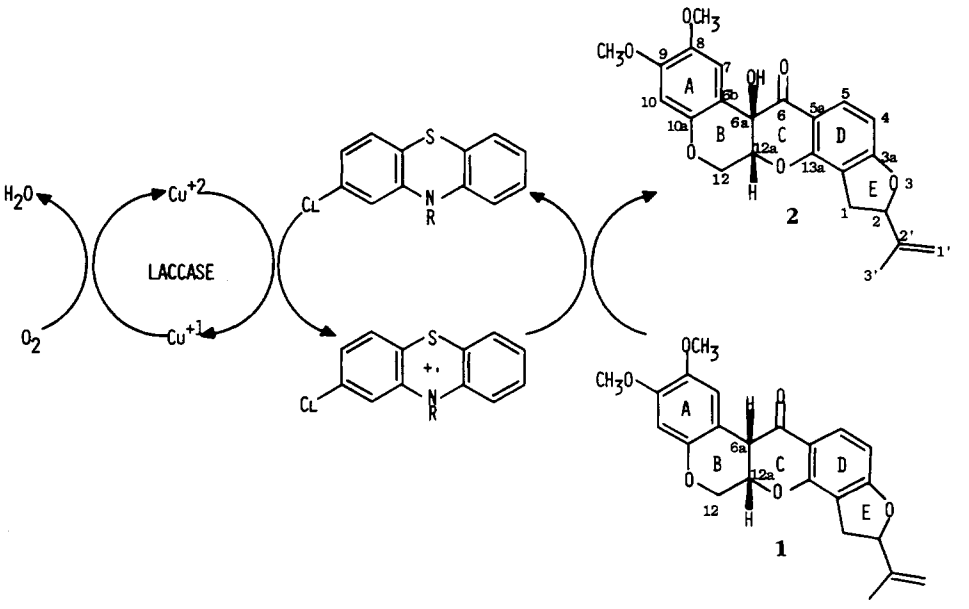

FIGURE 2. The pathway for the flow of electrons in the oxidation of rotenone (**1**) to rotenolone (**2**) by laccase from *Polyporus anceps*.

ACKNOWLEDGMENTS

We acknowledge financial support for this work through grant CA 13786-10. We also thank Dr. J.L. Sudmeier, Department of Chemistry, University of California at Riverside, and the Regional NMR Facilities of California Institute of Technology, Pasadena, California, for providing the 500 MHz spectra.

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Received 19 January 1984