One-Electron Oxidation of Vindoline and 16-*O*-Acetylvindoline Catalyzed by Peroxidase

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The mechanism of oxidation of the alkaloids vindoline (1) and 16-*O*-acetylvindoline (**1a**) was examined by use of the reversible redox cycle of horseradish peroxidase (HRP). Oxidation of 1 by HRP resulted in the formation of the enamine dimer **5**. The highly reactive radical cation species **2** is an implied intermediate in the oxidation process. During the reaction, HRP-I was reduced to HRP-II by abstraction of an electron from vindoline. The vindoline radical thus formed eliminates a second electron and a proton to produce a highly reactive iminium derivative which undergoes intramolecular etherification and dimerization. Oxidation of 16-*O*-acetylvindoline (**1a**) by HRP-I results in the production of an iminium derivative **3a** concomitant with the formation of HRP-II. The iminium **3a** was isolated and characterized and was converted into monodeuterated **1a** by reduction with $NaBD_4$. The stoichiometry (HRP-II)/(substrate) was determined to be 4.77 ± 0.17 for vindoline and 2.27 ± 0.20 for 16-*O*-acetylvindoline. The enamine dimer also reduced HRP-I to form HRP-II, but the stoichiometry of this reaction was variable.

Vindoline (1) is an Aspidosperma alkaloid found abundantly in *Catharanthus roseus* plants. The compound is interesting because of its biosynthetic relationship to many other monomeric and dimeric alkaloids and because it is a member of a broader array of natural and synthetic nitrogen heterocycles which may enter into and elicit physiological effects on living systems.[1,2] Vindoline (1) and and its chemical derivative 16-*O*-acetylvindoline (**1a**) have served as useful model compounds for elaborating biochemical mechanisms by which nitrogen heterocycles are converted into other molecules including biologically reactive intermediates.[3-5] Investigations of these alkaloids with the bacterium *Streptomyces griseus*,[5] copper oxidases including human serum ceruloplasmin and plant and fungal laccases,[3] and the chemical oxidizing agent 2,3-dicyano-4,5-dichlorobenzoquinone (DDQ)[4] revealed that the 3-position of Aspidosperma alkaloids is susceptible to oxidation. A pathway for vindoline oxidation reactions was proposed (Figure 1). Previous work permitted the actual isolation and complete physical characterization of compounds **3a**,[4] **4**,[6] and **5**. We now report on the use of the well-known catalytic cycle of horseradish peroxidase to confirm the involvement of a free-radical species in vindoline oxidations.

Horseradish peroxidase (EC 1.11.1.7, donor H_2O_2 oxidoreductase) is a monomeric protein containing a single protoporphyrin IX heme functional group. The iron porphyrin moiety of HRP may exist in any of five known redox states ranging from 2+ to 6+.[7-9] Intermediate redox forms of 3+, 4+, and 5+ have been identified and the oxidative cycle of HRP has been well defined. The native enzyme contains a high-spin porphyrin iron(3+) moiety which undergoes two-electron oxidation upon reaction with hydrogen peroxide or organic peroxides. The resulting enzyme known as HRP-I has been identified as a low-spin porphyrin iron(4+) π cation radical species by visible, Mössbauer, ESR, and ENDOR spectral measurements.[10,11]

HRP-I catalyzes one-electron oxidation reactions with suitable substrates, resulting in the formation of a red redox species of the enzyme designated as HRP-II. HRP-II contains a porphyrin iron(4+) system which may be converted back to native HRP by one-electron reduction. Compounds which serve as substrates for HRP-I oxidations usually form free-radical intermediates which may undergo a variety of further chemical reactions.[12,13] The scope of the reactions catalyzed by peroxidases through HRP-I and other forms of the enzymes has been systematically investigated.[14-17] This paper describes the reactions of vindoline, 16-*O*-acetylvindoline, and an enamine dimer with HRP-I.

Results

Identification of Products Formed by HRP Oxidation of Vindoline and 16-*O*-Acetylvindoline. (a) Formation of the Dimer 5 from Vindoline (1). Preparative-scale incubation of HRP with vindoline resulted in the formation of a single product in 20% yield (estimated by TLC). The isolated product was examined by mass spectrometry and proton NMR spectroscopy for structure elucidation. Mass spectral (FAB) analysis of the product indicated a protonated molecular ion at m/e 909 for $C_{50}H_{61}N_4O_{12}$. The high-field proton NMR spectrum of the HRP-oxidation product gave the following (ppm): 0.822 (6 H, dt, 18-H), 1.347 (4 H, m, 19 H), 1.6–1.90 (4 H, m), 1.96 (3 H, s, $COCH_3$), 2.0094 (3 H, s, $COCH_3$), 2.72 (3 H, s, NCH_3), 2.79 (3 H, s, NCH_3), 3.7538 (6 H, overlapping Ar OCH_3), 3.771 (3 H, s, $COOCH_3$), 3.781 (3 H, s,

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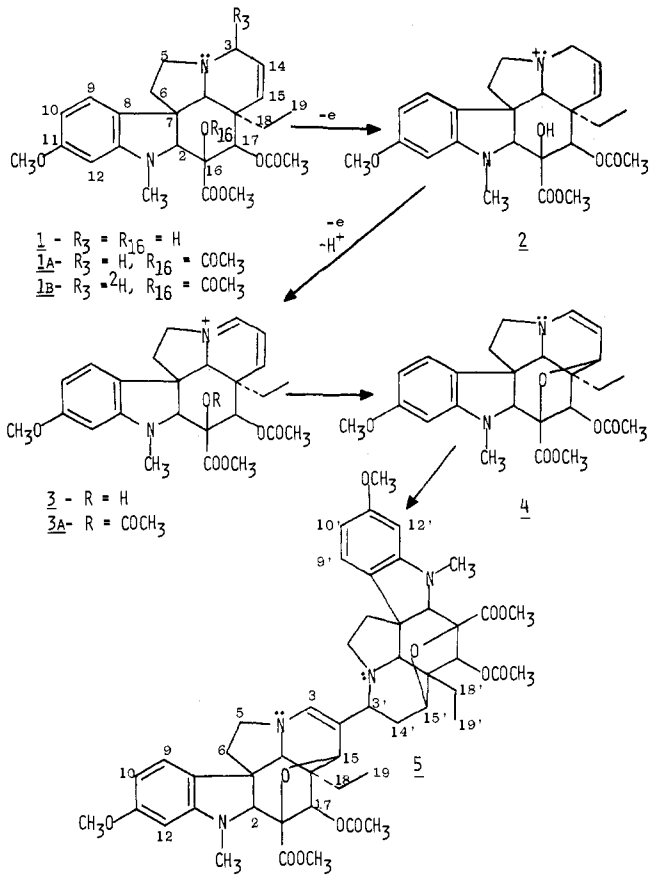

Figure 1. The pathway of oxidation of vindoline (1) and 16-*O*-acetylvindoline (1a).

$COOCH_3$), 4.058 (1 H, m, 15'-H), 4.281 (1 H, 15-H), 5.358 (1 H, s, 17-H), 5.402 (1 H, s, 17-H), 6.035 (1 H, d, J = 2.1 Hz, 12-H), 6.077 (1 H, d, J = 2.1 Hz, 12-H), 6.216 (1 H, s, 3-H), 6.278 (2 H, q, J = 8.2, 2.16 Hz, 10-H), 7.006 (1 H, d, J = 8.21 Hz, 9-H), 7.081 (1 H, d, J = 8.21 Hz, 9-H). These proton NMR and mass spectral data are identical with those recorded previously for the enamine dimer **5**.[5]

(b) Formation of the Iminium Derivative 3a of 16-*O*-Acetylvindoline (1a). The single polar product formed (100% conversion) during the HRP oxidation of **1a** was isolated by preparative layer chromatography and examined by chemical-ionization mass spectrometry: m/e (percent relative abundance) 498 (30.2), 4.97 (79.1), 437 (100), 296 (6.4), 295 (16.5), 188 (38.1), 174 (9.3), 135 (11.5), 122 (16.5), 121 (2.8). This pattern is identical with that obtained for **3a** produced by enzymatic, chemical, and bacterial oxidation of **1a**.[4] The position of the iminium ion double bond was determined by reducing the iminium metabolite with sodium borodeuteride in deuteriomethanol. The reduced HRP-I metabolite **1c** was chromatographically identical with 16-*O*-acetylvindoline. The electron-impact mass spectrum of **1c** gave the following: m/e (percent relative abundance) 499 (1.1), 498 (0.2), 297 (12.6), 189 (49), 175 (17.1), 174 (42.7), 136 (100), 135 (66), 122 (53.5), 121 (31.4). These mass spectral data are completely consistent with the structure of the deuterium-containing metabolite as **1c**.[4]

Oxidations of Vindoline (1), 16-*O*-Acetylvindoline (1a), and the Dimer 5 by HRP-I. Solutions of HRP (native enzyme) in pH 6.8, 0.1 M phosphate exhibited a maximum absorbance in the visible spectrum at 403 nm.[18]

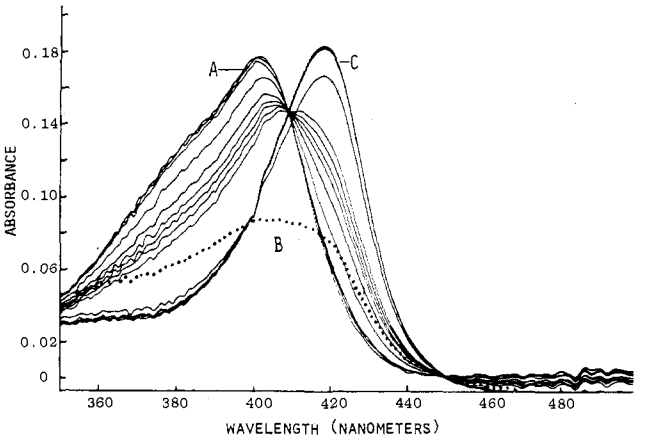

Figure 2. Visible absorption spectra of HRP native enzyme (403 nm), HRP-I (410 nm), and HRP-II (418 nm). Cuvettes contained 3-mL mixtures of HRP (4.7 μM) (A); HRP plus equimolar hydrogen peroxide (B); HRP, equimolar hydrogen peroxide, and vindoline (7.0 μM) (C). Spectra were recorded every 15 s.

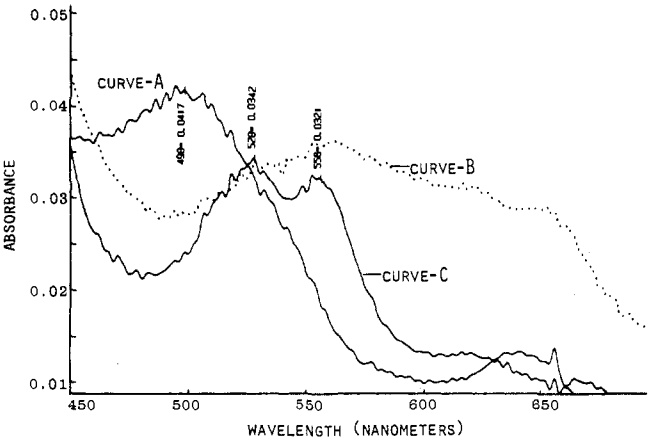

Figure 3. Visible absorption spectra of HRP (curve A), HRP-I (curve B), and HRP-II (curve C). Conditions were the same as those given in Figure 2.

Upon addition of hydrogen peroxide to the native enzyme solution, the absorption spectrum was broadened with a maximum of 410 nm, typical for the Soret band displayed by HRP-I.[19,20] Addition of either 1 (7 μM), 1a (6 μM), or **5** (4 μM) to the HRP-I enzyme reaction mixture resulted in a shifting of the absorption maximum of the solution to 418 nm (Figure 2). This absorbance is typical for the Soret band displayed in the visible spectrum by HRP-II. Incubation of substrate-containing reaction mixtures for longer periods of time resulted in a shift to 403 nm in the visible absorption spectrum, the absorption maximum of native HRP (Figure 2).

Various redox forms of HRP also exhibit absorption differences in the 450–750-nm wavelength range (Figure 3).[21] For example, HRP (native enzyme) absorbs light at 498 nm (Figure 3A), while HRP-I contains a broad and ill-defined absorption band between 500 and 700 nm typical for those shown by π cation radical metalloproteins[21] (Figure 3B). HRP-II, on the other hand, displays three absorption maxima in this region at 528, 556, and 664 nm (Figure 3C). When 1, **1a**, or **5** were added to incubation mixtures containing HRP-I, clear-cut changes

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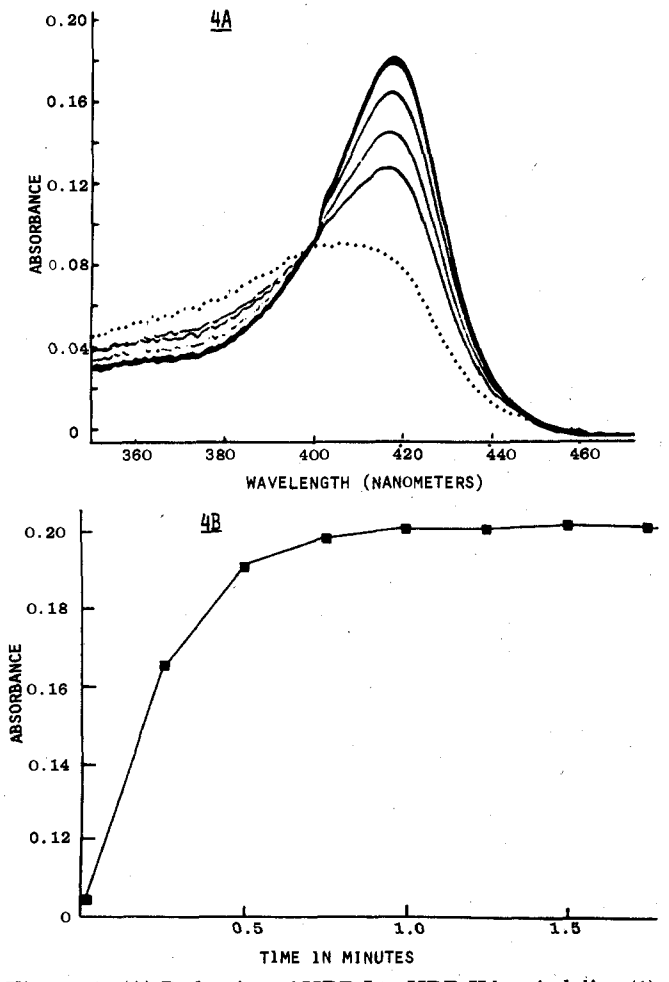

Figure 4. (A) Reduction of HRP-I to HRP-II by vindoline (1). The reaction mixture consisted of 3 mL of 0.1 M sodium phosphate, pH 6.8, containing HRP-I (4.7 μM). The dotted line represents the absorption spectrum of HRP-I before vindoline. Vindoline (0.7 μM) was added and complete spectra were recorded every 15 s. (B) The rate of formation of HRP-II as evidenced by the increase in absorbance at 418 nm after addition of vindoline to an HRP-I mixture. Reaction conditions were identical with those used in Figure 4A.

in the visible spectrum of HRP redox forms were displayed. The broad absorption band attributable to HRP-I was replaced by the three peaks characteristic for HRP-II. These changes were recorded for incubations conducted at either pH 6.8 or pH 5.6.[22]

The Stoichiometry of Alkaloid Oxidations by HRP-I. The stoichiometries of alkaloid oxidations by HRP-I were determined by preparing HRP-I solutions of known molarity and by addition of 1, **1a**, or **5** to the enzyme reaction mixtures. The amount of HRP-II formed during enzyme oxidation reactions was calculated by measuring the increase in absorbance at 418 nm, and the results of these experiments are shown in Figures 4A and 4B. Figure 4A shows that addition of 1 to HRP-I results in a gradual shifting of the absorption maximum from 410 to 418 nm. This effect can be quantified for all three substrates. Figure 4B displays the increase in absorbance at 418 nm obtained when HRP-I (4.7 μM) is reacted with 1 (0.7 μM). The reaction is essentially complete 30 s after vindoline is added to the reaction mixture. The calculated ratio of moles of HRP-II formed to moles of vindoline oxidized is 4.8 ± 0.2 (n = 4). In similar experiments where **1a** or **5** were used as substrates for the reduction of HRP-I, the stoichiometries obtained were 2.3 ± 0.2 and 7.3 ± 0.36, respectively.

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Discussion

The oxidation of vindoline (1) and 16-*O*-acetylvindoline (**1a**) by copper oxidases, DDQ as a chemical mimic of the copper oxidases, and the enzyme systems of *S. griseus* all follow the pathway illustrated in Figure 1.[3,5] Extensive evidence permitted the complete chemical characterization of all compounds shown in Figure 1 except for **2**. However, no direct evidence to support the involvement of radical cation intermediates such as **2** or **2a** in this pathway could be obtained.

In order to elucidate the mechanism of the initial oxidation step in this pathway, the oxidative capabilities of an enzyme system with well-defined characteristics were exploited. The peroxidases are well-known for their abilities to oxidize a wide range of substrates by peroxidatic, oxidatic, and catalatic mechanisms. Thus, 1, **1a**, and **5** were examined as possible substrates with this enzyme.

Preparative-scale incubations of 1 and **1a** with HRP and an excess of hydrogen peroxide resulted in the formation of new products. These were isolated by preparative layer chromatography and characterized by spectral and chromatographic analyses and by comparisons with authentic standards.[3-5] Vindoline was transformed into a less polar substance (TLC) chromatographically similar to the enamine dimer **5**.[3,5] The protonated molecular ion peak at m/e 909 was obtained with both chemical-ionization and FAB mass spectrometry. All of the signals for **5** were also clearly evident in the 360-MHz NMR spectrum of the HRP-oxidation products. Characteristic among these were the two *N*-methyl group resonances at 2.72 and 2.79 ppm, two *O*-acetyl methyl group signals at 1.96 and 2.01 ppm, the singlet olefinic signal for proton 3 H at 6.216 ppm, and signals for the methine ether protons of positions 15 and 15′ at 4.058 and 4.284 ppm.[3,5] This NMR spectrum, together with the mass spectral data and the chromatographic mobility comparisons with authentic **5**, conclusively demonstrated that HRP had transformed 1 into the enamine dimer **5**.

16-*O*-Acetylvindoline (**1a**) was also a substrate for HRP. With **1a**, the iminium metabolite **3a** accumulated in incubation mixtures as a trapped intermediate because subsequent intramolecular etherification (**3** to **4**, Figure 1) between the alcohol functional group at position 16 and the conjugated iminium moiety through position 15 was blocked. In earlier work with other enzymes, **3a** was isolated and characterized by mass spectrometry and proton NMR spectroscopy.[4] Reduction of iminium **3a** with sodium borodeuteride in deuteriomethanol resulted in the quantitative formation of 3α-deuterio-16-*O*-acetylvindoline (**1b**). With all enzyme and chemical systems studied, the deuterium atom was introduced specifically into the 3α-position. An added advantage of the deuteration procedure is that the identity of **1b** was readily confirmed by chromatography and mass spectrometry.[4] The isolated polar product obtained from HRP oxidation of **1a** gave a protonated molecular ion peak at m/e 498 in the chemical-ionization mass spectrum. The polar metabolite was reduced with sodium borodeuteride to provide a new compound chromatographically identical with **1a**. The molecular ion of the reduced product in the electron-impact mass spectrum was m/e 499, indicating the presence of a single deuterium atom in the structure. Other key fragments in the mass spectrum were at m/e 297, 136, and 122, all of which support the location of the deuterium

atom at position 3 as shown in **1b**. Thus, the structure of the polar metabolite formed by HRP oxidation of **1a** is **3a**.

To unravel the mechanism of the initial oxidation step(s) during the formation of **3a** and **5** by HRP, two different approaches were initially explored. These were the direct measurement of radical cation intermediates and the use of the well-defined characteristics of various HRP-redox species. Several attempts were made to detect radical species in enzyme reaction mixtures containing 1 and **1a**. Direct measurements by ESR spectroscopy require radical species like **2** with sufficiently long half-lives to permit spectral observation. Radical trapping may also be used to provide indirect evidence for the formation of unstable radical intermediates in enzyme reaction mixtures.[23] For unknown reasons, neither of these approaches were successful. Attention was then focused on exploiting the characteristics of different redox species of HRP to define the initial oxidation step.

Addition of hydrogen peroxide to solutions of native HRP results in the formation of HRP-I, a porphyrin iron(4+) system which includes a stabilized radical cation species.[10,11] HRP-I catalyzes one-electron oxidation reactions with substrates to form a variety of products, including HRP-II, the reduced form of the enzyme. Reactions of this type may be followed by measuring the increase in absorbance at 418 nm due to the formation of HRP-II. However, an additional redox form of the enzyme known as HRP-III also absorbs light at 418 nm. HRP-II may be distinguished from HRP-III at higher wavelengths, since HRP-II absorbs light at 527, 555, and 560 nm while HRP-III absorbs light at 545, 583, and 673 nm. An interpretation of HRP-I oxidation reactions may also be complicated by the further reduction of HRP-II back to the native enzyme, which results in a shift of the absorption maximum from 418 back to 403 nm. These complications are avoided by (1) addition of equimolar amounts of hydrogen peroxide to native HRP to form HRP-I and (2) by addition of limited amounts of substrates to HRP-I.

Vindoline (1) and 16-*O*-acetylvindoline (**1a**) were added to solutions of HRP-I prepared in this manner. The shift in visible absorption from 410 to 418 nm (Figures 2 and 3) and at higher wavelengths clearly showed that HRP-II was the only redox form of the enzyme produced as the substrates were oxidized. These results provide the first evidence to suggest that the initial step in the oxidation of 1 and **1a** occurs by one-electron oxidation to form **2** and **2a**. The iminium cations **3** and **3a** would then form by the elimination of a second electron and a proton from the adjacent 3-position. However, the evidence presented cannot exclude the possibility of an intitial hydrogen atom abstraction from position 3 to generate a resonance stabilized allylic radical followed by a second electron oxidation to generate iminium compounds **3** and **3a**.

The measured stoichiometries for 1 and **1a** oxidations revealed that 4.8 ± 0.2 mol of HRP-II are formed for every mole of vindoline oxidized and that 2.3 mol of HRP-II are formed when 16-*O*-acetylvindoline is the substrate. These results suggest that the enamine dimer **5** also serves as a reducing substrate for HRP-I. The dimer **5** possesses several sites which might be susceptible to HRP-I oxidation including positions 3, 3′, 5, 5′ (Figure 1). When **5** was examined as a possible substrate for HRP-I, the measured stoichiometries were variable and exhibited a maximum value of 7.3 ± 0.4. In the case of **1a**, HRP-I oxidations can only proceed to the iminium **3a**, thus accounting for the observed differences in stoichiometry between the two alkaloid substrates. A scheme to account for these observations is presented in Figure 5.

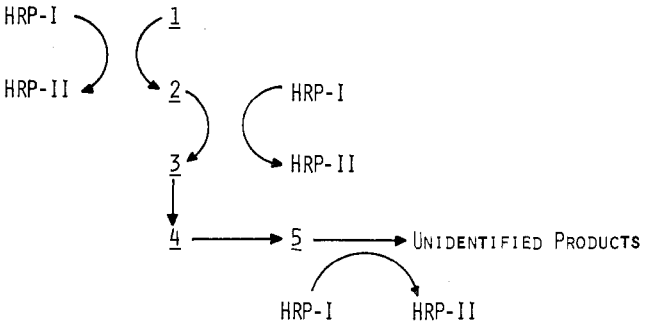

Figure 5. A proposed scheme for the oxidation of vindoline (1) and 16-*O*-acetylvindoline (**1a**) by HRP-I.

The present study confirms the involvement of free-radical oxidative mechanisms in enzymatic transformations of the *Catharanthus* alkaloid vindoline and its 16-*O*-acetyl derivative. Vindoline is biosynthetically related to a large number of other monomeric and dimeric alkaloids possessing similar chemical functionalities. Some of these compounds have been widely used in the clinical treatment of human cancer.[24] It is logical to assume that similar free-radical oxidations will be observed among this group of nitrogen heterocycles. It is noteworthy the peroxidases have been utilized as catalysts in achieving the transformation of other alkaloids including dehydrovinblastine, boldine, morphine, lysergic acid derivatives,[25] and ellipticines.[12,13] Detailed mechanistic studies were accomplished with ellipticines for their reaction with peroxidases, but much less is known of the intermediates and pathways followed in oxidations of other alkaloids. Methods established in this work are being exploited in order to elucidate mechanistic information for bioconversions with other monomeric and dimeric *Catharanthus* alkaloid substrates.

Experimental Section

Instrumentation. Proton magnetic resonance (^{1}H NMR) spectra were obtained at 60 MHz with a Varian EM360 instrument and at 360 MHz with a Bruker WM-360 FTQ NMR instrument using CD_3OD as solvent and tetramethylsilane as an internal standard. Low-resolution mass spectra were obtained with a Ribermag R-10-10C, Nermag-S-a, France spectrometer. Chemical-ionization mass spectra were obtained on the same instrument using ammonia as the reagent gas. Fast atom bombardment mass spectrometry (FAB) was carried out on a Kratos MS-50 triple analyzer using xenon as the carrier gas with samples dissolved in glycerol. Ultraviolet and visible absorption spectra were obtained at controlled temperatures in stirred cells with a Hewlett-Packard Model 8450A UV/visible spectrophotometer linked to an HP-85 microcomputer, an HP7470A plotter, an HP82901M flexible disc drive, and an HP89100A temperature controller.

Materials. Vindoline (1) was obtained from Eli Lilly and Co., Indianapolis, IN. The iminium **3a** and the enamine dimer **5** were authentic standards available from previous work in our laboratory.[4,5] 16-*O*-Acetylvindoline (**1a**) was prepared as described.[4] Horseradish peroxidase, type VI (A_{403}/A_{280} = 3.0), was purchased from Sigma Chemical Co., St. Louis, MO. The enzyme displayed a single band on $NaDodSO_4$ polyacrylamide gel electrophoresis.[26] Sodium borodeuteride was obtained from Sigma Chemical Co.

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Chromatography. Thin-layer chromatography (TLC) was performed on 0.25- or 0.5-mm layers of silica gel GF254 (Merck) prepared on glass plates. After air-drying, TLC plates were activated at 120 °C for 30 min prior to use. Solvent systems used were as follows: A, ethyl acetate–methanol (3:1), vindoline (1) R_f 0.7, 16-*O*-acetylvindoline (**1a**) R_f 0.82, dimer **5** R_f 0.8, and the iminium compound **3a** R_f 0.1; and B, ethyl acetate–benzene (4:1), vindoline (1) R_f 0.18, dimer **5** R_f 0.34. Compounds were detected on developed chromatograms by fluorescence quenching under 254-nm UV light or with cerium(IV) ammonium sulfate (1% w/v in 50% v/v phosphoric acid).[27]

Oxidation of Vindoline (1) and 16-*O*-Acetylvindoline (1a) Catalyzed by Horseradish Peroxidase. Analytical-scale oxidations of 1 and **1a** were carried out in 10 mL of 100 mM sodium phosphate (pH 6–7.5) in 125-mL Delong flasks containing 1 mg of HRP (330 purpurogallin units), 0.8 mL of 1.7 mM hydrogen peroxide, and 4 mg of the substrate dissolved in 0.1 mL of methanol. The mixtures were incubated on a New Brunswick Scientific Co., G-24 gyrotory shaker at 250 rpm and 28 °C and 3-mL samples were withdrawn every 15 min, adjusted to pH 9.5 with ammonium hydroxide, and extracted with 1.5 mL of ethyl acetate. A 30-μL aliquot of the extract was applied to 0.25-mm silica TLC plates and developed with solvent system A for **1a** or solvent system B for 1. Product formation was observed after 45 min. No products were formed when alkaloid substrate or hydrogen peroxide were deleted from the reaction mixture. The pH optimum of the complete reaction mixtures was 6.8.

Preparative-Scale Production of Iminium 3a from 16-*O*-Acetylvindoline (1a). 16-*O*-Acetylvindoline (**1a**; 40 mg in 2 mL of methanol) and HRP (10 mg, 3300 units) were added to 100 mL of pH 6.8, 0.1 M sodium phosphate together with 8 mL of 1.7 mM hydrogen peroxide. The reaction mixture was divided among ten 125-mL Delong flasks and incubated with shaking for 1 h. TLC monitoring of the reaction as before indicated that a polar metabolite (R_f 0.1, solvent system A) with chromatographic properties identical with iminium derivative **3a** was formed after 45 min of incubation. Excess hydrogen peroxide remaining in the incubation mixture after 1 h was reduced by adding 0.5 mL of a solution of 0.2 M sodium thiosulfate to each flask.[28] One hour after addition of the reductant, the mixtures were adjusted to pH 9.5 with ammonium hydroxide solution and extracted three times with 100 mL of ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. This residue was dissolved in methanol and applied to 0.5-mm TLC plates and developed with solvent system A. The band centered at R_f 0.1 was scraped from the plates, and the products were eluted by several rapid washes with ethanol. The collected eluates were concentrated and dried under vacuum to provide 10 mg of the iminium **3a**.

The polar iminium derivative **3a** (9 mg) was dissolved in 5 mL of CH_3OD, and 50 mg of $NaBD_4$ was added to the solution. The reduction was complete in 30 min and the product formed was identical with 16-*O*-acetylvindoline (**1a**) by TLC (R_f 0.82, solvent A). The reduction mixture was evaporated to dryness under a stream of nitrogen, and the solid was dissolved in 10 mL of water and extracted with three 10-mL portions of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous Na_2SO_4, filtered, concentrated under nitrogen, and dried under high vacuum to give 8 mg of **1a**. This compound was subjected to mass spectral analysis.

Production of the Enamine Dimer 5 by HRP Oxidation of Vindoline (1). Vindoline (1; 100 mg in 1 mL of methanol) and HRP (25 mg, 8250 units) were added to 250 mL of pH 6.8, 0.1 M sodium phosphate and 20 mL of 1.7 mM hydrogen peroxide solution. The reaction was carried out as described before, and TLC analysis indicated that a new product formed in approximately 20% yield. The reaction mixture was extracted with ethyl acetate, concentrated, and applied to 0.5-mm TLC plates and developed in solvent system B. The band centered at R_f 0.34 was eluted from silica gel to provide 14 mg of pure product. This compound was subjected to NMR and mass spectral analyses.

Determination of Concentrations of Hydrogen Peroxide, HRP Native Enzyme, HRP-I, HRP-II, and Substrates. Hydrogen peroxide solutions were prepared by diluting commercially available 30% hydrogen peroxide with distilled water immediately before addition to enzyme reactions. Concentrations were standardized by measuring the UV absorption of diluted hydrogen peroxide solutions at 240 nm, with $\epsilon = 43.6\ M^{-1}\ cm^{-1}$,[29] and these solutions were stable for 2 h.

Horseradish peroxidase (HRP) solutions were prepared by dissolving the enzyme in 0.1 M sodium phosphate over the range of pH 6–7.6 or in pH 5.6, 0.2 M sodium acetate. Enzyme concentrations were determined spectrophotometrically by measuring absorptions at 403 nm, with $\epsilon = 9.0 \times 10^4\ M^{-1}\ cm^{-1}$.[30] For stoichiometry measurements, concentrations of HRP-I and HRP-II were determined by measuring the absorbances of solutions at 418 nm, with $\epsilon = 3.7 \times 10^4\ M^{-1}\ cm^{-1}$ and $\epsilon = 8.7 \times 10^4\ M^{-1}\ cm^{-1}$, respectively.[30]

Concentrations of substrates added to reaction mixtures were determined by UV measurements of ethanolic stock solutions of the alkaloids with use of the following molar extinction coefficients: vindoline (1), $\epsilon = 7.4 \times 10^3\ M^{-1}\ cm^{-1}$ at 250 nm;[4,5] 16-*O*-acetylvindoline (**1a**), $\epsilon = 7.2 \times 10^3\ M^{-1}\ cm^{-1}$ at 252 nm;[4,5] and the enamine dimer **5**, $\epsilon = 1.8 \times 10^4\ M^{-1}\ cm^{-1}$ at 249 nm.[5]

The Stoichiometry of Alkaloid Oxidations by HRP-I. All reactions were conducted in 3-mL volumes of pH 6.8, 0.1 M sodium phosphate in stirred quartz cuvettes. Native HRP was used in concentrations of $(4.1–4.7) \times 10^{-6}$ M and was carefully oxidized to HRP-I by titration of the enzyme solution with an equimolar amount of hydrogen peroxide solution. The concentration of HRP-I was confirmed by absorption measurements at 410 nm. The stoichiometry of the reaction between HRP-I and substrates was determined by the addition of 3–5 μL of a methanolic solution of 1, **1a**, or **5** (7×10^{-6} M) to the cuvette containing the HRP-I. Control experiments showed that the substrates were all stable in methanol and that the addition of this amount of methanol did not alter the concentration of HRP-I in the assay mixture. The increase in absorbance at 418 nm and the complete absorption spectra between 200–550 and 350–750 nm were recorded every 15 s and stored on flexible diskettes. Reaction stoichiometries were calculated with use of a molar absorptivity difference between HRP-I and HRP-II of $5.0 \times 10^4\ M^{-1}\ cm^{-1}$. Blank cuvettes in these experiments contained only the appropriate buffer. The spontaneous decay of absorbance due to HRP-I was monitored at 411 nm and was negligible during the typical reaction time period of 10 min.

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