Oxidations of Vincristine Catalyzed by Peroxidase and Ceruloplasmin

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The dimeric Catharanthus alkaloid vincristine (**1**) is oxidized to the same ring fission product in incubations with either horseradish peroxidase or the human serum copper oxidase ceruloplasmin. Horseradish peroxidase-catalyzed oxidation of vincristine requires hydrogen peroxide, whereas ceruloplasmin-catalyzed oxiation of vincristine requires chlorpromazine as a "shuttle oxidant". Preparative-scale incubations allowed for the production, isolation, structural characterization, and biological evaluation of the metabolite. The metabolite was identified as the heterocyclic ring cleavage product *N*-formylcatharinine (**5**). *N*-Formylcatharinine was 118 times less active than vincristine in an in vitro test against a human T-cell leukemic cell line. Therefore, these enzyme-catalyzed reactions lead to bioinactivation of vincristine.

The anticancer alkaloids, vincristine (VCR, **1**) and vinblastine (VLB, **2**, Figure 1), were originally obtained from the periwinkle plant *Catharanthus roseus* (L.) G. Don.[1,2] These alkaloids have been clinically used for the treatment of various cancers for more than three decades.[3] They function by preventing normal microtubule formation in dividing cells.[4,5] Despite much effort, little knowledge has been gained about in vivo metabolism, the origin of various cytotoxic effects, and the mechanism of action of these dimeric Catharanthus compounds. The simple ester hydrolysis product, 17-desacetylvinblastine (**3**, DAVLB), has been characterized as an in vivo metabolite of VLB. However, little else is known of the in vivo metabolism of Catharanthus alkaloids, likely because of their high tendency to rapidly deposit in adipose tissue, the very low doses involved, and the need for sufficient quantities of metabolites for complete spectral evaluation. For these reasons, we have focused attention on the in vitro metabolic/transformations of these compounds.[6,7] For VCR, no structures of metabolites from in vivo or in vitro studies have ever been reported. This is surprising because VCR is widely used in combination chemotherapy, since myelosuppression is not a dose-limiting side effect.[8]

This report describes in vitro metabolic transformation studies of VCR (**1**) by peroxidase and the human serum copper oxidase, ceruloplasmin. We describe a new VCR metabolite (**5**), which was isolated and identified by NMR, IR, and high-resolution mass spectral analyses. The biological activity of **5** against a T-cell leukemic cell line was compared with VCR (**1**) and VLB (**2**). The biotransformation reaction may be implicated in resistance to VCR in myeloblastic leukemic cells.[9]

Results and Discussion

Ceruloplasmin (EC 1.16.3.1) is a blue-copper oxidase protein that is present in vertebrate serum, accounting for up to 95% of the copper in the serum.[10] From in vitro experimental results, ceruloplasmin is thought to be involved in the regulation of circulating biogenic amines and catechols through its oxidase activity.[11] Copper oxidases catalyze the oxidation of xenobiotics such as the Catharanthus alkaloids in one of two ways. In the first, the substrate directly binds to the enzyme and undergoes initial one-electron oxidation. The second requires a shuttle oxidant[8,12,13] such as chlorpromazine that is enzymatically converted into a relatively stable cation radical, which leaves the enzyme-active site and catalyzes the oxidation of compounds such as VCR. In either case, cuprous copper at the enzyme active site is oxidized to cupric copper by molecular oxygen to initiate the oxidation process. These reactions and their features have been reviewed extensively.[8,12,13] Peroxidases are heme-containing glycoproteins that catalyze the oxidations of substrates when organic peroxides or hydrogen peroxide (H_2O_2) are present.[14] Horseradish peroxidase (HRP, EC. 1.11.1.7) is present in horseradish roots, and its biological function is to reduce H_2O_2 produced as a byproduct during enzymatic reactions.[15,16] HRP is readily available in resonably pure form and it is relatively stable. Therefore, it is the most extensively studied peroxidase, and it is often used as a model enzyme for other peroxidases.[17] HRP undergoes a well-defined oxidative transformation when reacted with H_2O_2.[6,7,8] The native enzyme through the bound ferric iron at the heme center is oxidized to a powerful oxidant known as HRP-compound I. This form of the enzyme normally abstracts an electron from substrates in initiating oxidation reactions.

In our laboratory, the chemistry of peroxidase and ceruloplasmin-catalyzed VLB oxidations has been well characterized.[6,7] With these enzymes, VLB (**2**) undergoes an unusual C21′ and C20′ bond fission by a sequence involving one-electron oxidation at nitrogen and hydrogen elimination from the adjacent carbon to form an imonium intermediate that is hydrated to form a diol, the immediate precursor for a periodate-like enzymatic carbon−carbon fission reaction.

Analytical-scale incubations of VCR with peroxidase−H_2O_2 and ceruloplasmin using our well-established conditions consistently gave high yields of a single major alkaloid metabolite. The VCR metabolite (**5**, 27 mg, 37% yield) was isolated, chromatographically purified

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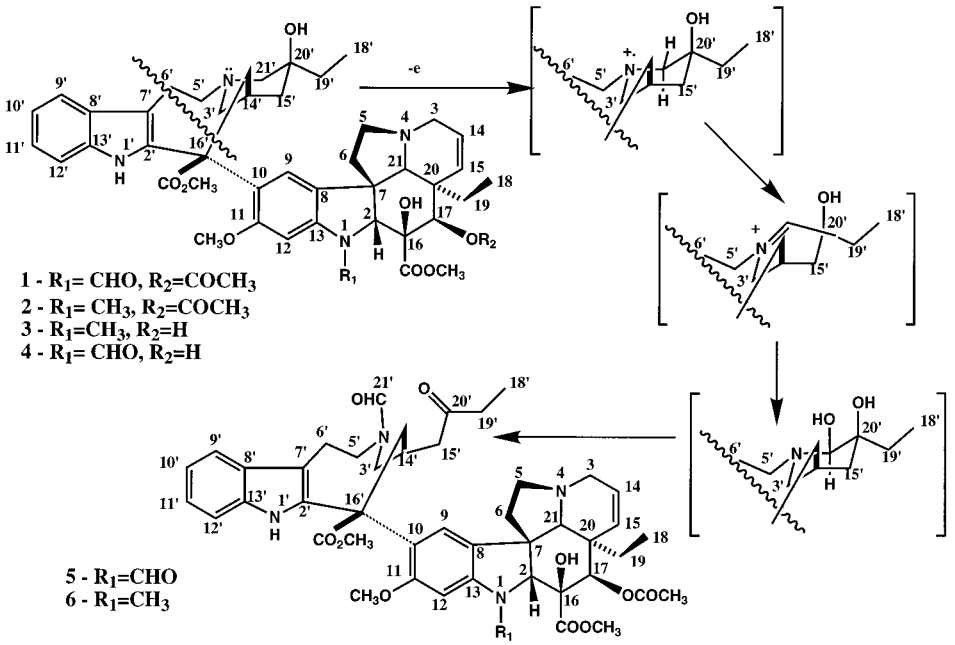

Figure 1. Proposed pathway for HRP−H_2O_2 and ceruloplasmin-catalyzed oxidative ring fission of VCR (**1**) to form N-formylcatharinine (**5**).

from incubations with HRP and H_2O_2, and subjected to spectral analysis. The metabolite gave a protonated molecular ion peak at m/z $(M + 1)^+$ 839.3881 (calculated 839.3872) for $C_{46}H_{55}N_4O_{11}$ by HRMS. This molecular ion is 14 mass units higher than that for VCR (825.4089 for $C_{46}H_{57}N_4O_{10}$).

The ^{1}H NMR spectrum of **5** ($CDCl_3$, 600 MHz) was compared with the spectra of VCR and other known Catharanthus dimers. The ^{1}H NMR spectral properties of 17-desacetylvincristine (DAVCR, **4**) free base[18] are similar to those for VLB,[19,20] except for signals for H-2 and the aromatic singlets for H-9 and H-12. These are all shifted downfield by nearly 1 ppm due to the N−CHO group of **4**, which is attached to the Aspidosperma dihydroindole aromatic ring and the adjacent position 2. The ^{1}H NMR spectra of VCR base ($CDCl_3$) and VCR·SO_4 (D_2O) were obtained and compared to the ^{1}H NMR spectral data for DAVCR (**4**).[18]

In general, the spectrum of the HRP−H_2O_2 metabolite (**5**) resembled the spectrum of VCR (**1**) and DAVCR (**4**) in that duplicate signals were present for many protons, especially those from the Aspidosperma ring. When they occurred, dual proton signals occurred in a 60/40 ratio, signifying the presence of rotameric N−CHO group isomers.[18] Rao et al. noted that, due to the orientations of the carbonyl group of vincristine derivatives, the anisotropy experienced by protons and carbons in the immediate vicinity of the formyl group leads to important chemical shift differences.[18] However, it was also observed that in the ^{1}H NMR spectrum small differences induced in other protons cause enlargements of the patterns and/or loss of resolution, thus precluding exact measurements of coupling constants in many instances.

Knowledge of the existence of rotameric forms of VCR base rendered the interpretation of ^{1}H NMR spectral data for VCR metabolite **5** possible. Despite the complexity of the metabolite spectrum, there were several distinct differences discernible versus the spectrum of VCR (**1**). The singlet proton signals for position 21′ at δ 3.3 in VCR·SO_4 and δ 2.8 in VCR (**1**) were absent in the spectrum of **5** and replaced by a new N−CHO signal at δ 7.52, which appeared as two singlets separated by J = 5.8 Hz. This signal is similar to the *N*-formyl signals in vinamidine[6] and 15′-hydroxycatharinine.[13] Even though the *N*-formyl group in **5** is on the Iboga ring, the doublet is likely due to the presence of two rotameric forms of the metabolite as with VCR (**1**) and DAVCR (**4**)[18] or due to *N*-formyl group rotamers on the Iboga ring.

^{13}C NMR spectroscopic comparisons of the metabolite and vincristine were much clearer. The ^{13}C NMR spectral assignments for VCR (**1**), DAVCR (**4**),[18] VLB (**2**),[21,22] and **5** were compared. In general, carbon assignments for VCR (**1**) are directly comparable to those for DAVCR (**3**) except for the 17-*O*-acetyl carbons. Carbon signals for the Aspidosperma ring system were comparable among all these compounds, as were most of the signals in the Iboga system. As with VCR (**1**), the metabolite spectrum exhibited twin signals for carbons at positions 2, 11, 12, 16, 17, the COO, and the N−CHO group.

For the metabolite **5**, significant spectral differences occurred, particularly in signals derived from or near the Iboga piperidine ring system. The signals for C-18′ (δ 42.1), C-20′ (δ 68.9), and C-21′ (δ 63.7) with VCR (**1**) were all absent in the spectrum of **5**. Two new carbon signals appeared at δ 211 (211.0, 211.3) and at δ 164.8 for new ketone and *N*-formyl groups, respectively. Downfield shifts occurred for C-18′ and C-19′ from δ 42.1 and δ 33.6 in **1** to δ 80.2 and δ 37.8 in **5**, respectively. These spectral results suggested that the new ketone group in **5** was at C-20′.

Mass, ^{1}H NMR, ^{13}C NMR spectral results all point to the structure of a metabolite in which carbon−carbon bond cleavage had occurred between positions 20′ and 21′ where these carbons were converted to ketone and N-formyl groups, respectively (Figure 1). These changes are directly comparable to the structural changes catalyzed by HRP with VLB[6] and leurosine.[13]

Ceruloplasmin-catalyzed oxidation of VCR (**1**) produced a metabolite chromatographically similar to **5**. As

Table 1. IC_{50} Values of VCR (**1**), *N*-Formylcatharinine (Vincristine Metabolite **5**), Catharanine (**6**), and VLB (**2**) vs. the CEM-CCRF, T-Cell Leukemic Cell Line

compd tested	IC_{50}, mg/mL	compd tested	IC_{50}, mg/mL
VCR (**1**)	0.06	*N*-formylcatharinine (**5**)	7.10
VLB (**2**)	0.03	catharinine (**6**)	7.70

previously discovered with VLB, the ceruloplasmin oxidation reaction required chlorpromazine (CPZ) as a shuttle oxidant.[7,12,23,24] Pure metabolite was obtained in 18% yield (13 mg) by preparative-scale ceruloplasmin oxidation of VCR and subjected to spectral analysis. The protonated molecular ion at *m/z* 839.3872 for $C_{46}H_{55}N_4O_{11}$ was identical with that obtained for the HRP metabolite **5**. ^{1}H NMR spectral comparisons of the ceruloplasmin metabolite **5** revealed that signals for protons affected by rotameric isomers and all others were identical with those obtained in the spectrum of HRP-catalyzed VCR metabolite **5** (data not shown). Furthermore, the band at 1662 cm^{-1} in the IR ($CHCl_3$) spectrum of **5** confirmed the presence of a new *N*-formyl group that is absent in the IR spectrum of VCR.

Results obtained with HRP$-H_2O_2$ and copper oxidase oxidations of **1** show that ring-cleavage reactions are similar to those previously observed with leurosine[13] and VLB.[6] On the basis of our earlier work with VLB and leurosine, we suggest a mechanism for peroxidase-mediated oxidation of **1** to **5** as shown in Figure 1. Initial one-electron oxidation of the Iboga-Nb′ atom followed by hydrogen loss at the adjacent carbon (21′) produces a putative imonium derivative. Nucleophilic addition of H_2O[6] affords a vicinal diol that undergoes periodate-like diol cleavage to give **5**. Further mechanistic details including the activation of the alkaloid, the involvement of H_2O, H_2O_2, and O_2 in the reaction, and spectral observations of the enzyme active site as it oxidizes these alkaloids are the subjects of another report.

The biological activity of metabolite **5** was examined in the Catharanthus alkaloid-sensitive, CEM-CCRF, T-cell leukemic test system, and the results are shown in Table 1. The IC_{50} value for **5** was 118 and 237 times greater than those of VCR and VLB[7,8] and was comparable to the IC_{80} for catharanine (**6**). Therefore, oxidation by peroxidase or ceruloplasmin leads to the inactivation of VCR.

Schlaifer et al. demonstrated that myeloperoxidase (MPO) is linked to VCR resistance in human myeloblastic leukemia cells.[9] An important difference between acute myeloblastic leukemia and acute lymphoblastic leukemia is in their sensitivities to VCR. Both MPO described by Schlaifer et al. and HRP used in this study are heme-centered peroxidases that function oxidatively by a similar mechanism. In their work, Schlaifer et al. showed that both HRP and MPO catalyzed VCR's H_2O_2-dependent, oxidative breakdown. Furthermore, they showed that the degree of MPO activity in cell lines correlated in a positive way with cell line resistance to VCR cytotoxicity. The structures of VCR metabolites were never determined in this work. Our study demonstrates the specific chemical transformations of vincristine catalyzed by peroxidases, which are likely responsible for inactivation of VCR by MPO-containing cells. Thus, this work identifies the chemical changes involved in the mechanism of VCR resistance in such cell lines.

Selective inhibition of MPO or the protection of VCR by coadministration of antioxidants such as ascorbate or tocopherols could ameliorate the degradation of VCR by peroxidative enzymatic transformations.

Experimental Section

General Experimental Proceedures. LRMS were obtained using CI with a NERMAG R10-10C mass spectrometer (70 eV ionization potential). HRMS were recorded with a ZAB-280HF mass spectrometer or a Kratos MS-80 triple-analyzer mass spectrometer. ^{13}C NMR spectra were recorded in $CDCl_3$ for free base forms of VCR (**1**) and VCR metabolite (**5**) at 90.56 MHz with a Bruker WM-360 FT spectrometer using a Bruker Aspect 2000 computer connected with a Houston plotter. ^{1}H NMR spectra were acquired at 600.14 MHz with a Bruker AMX600 FT NMR spectrometer using a Bruker X32 computer or 360.13 MHz with a Bruker WM-360 FT NMR spectrometer using a Bruker Aspect 2000 computer. Chemical shift values are reported in ppm. IR spectra were recorded in $CHCl_3$ (0.1 mm NaCl cells) using a Nicolet 8DXB FT-IR spectrometer.

Chromatography. TLC analyses were performed on 0.28- or 0.8-mm-thick Si gel GF_{254} plates (Merck) in which the R_f values of VCR (**1**) and its ceruloplasmin- or HRP-produced metabolite (**5**) were 0.4 and 0.6, respectively. Plates were developed with $CHCl_3$−MeOH (10:1, v/v), and spots were visualized by UV fluorescence-quenching at 254 nm by spraying the developed plates with Dragendorff's reagent or keeping the plates in an iodine-vapor saturated chamber.

Materials. VCR·SO_4 (**1**) was obtained from Eli Lilly and Co., Indianapolis, IN, and OMNICHEM, PRB, Belgium, and its physical and spectral properties were compared with those reported for VCR and other Catharanthus alkaloids:[18] HRMS *m/z* (M + 1) 825.4089 for $C_{46}H_{57}N_4O_{10}$; ^{1}H NMR ($CDCl_3$, 360 MHz) δ 8.17 and 8.76 (NCHO), 8.06 (1H, NH), 7.54 (1H, d, J = 8.10 Hz, H-9′), 7.17 (1H, H-12′), 7.13 (2H, H-10′, H-11′), 6.93, 7.74 (1H, H-12), 6.80 and 6.88 (1H, H-9), 5.91 (1H, m, H-14), 5.41 (1H, d, J = 10.4 Hz, H-15), 5.22 and 5.28 (1H, H-17), 4.51 and 4.74 (1H, H-2), 3.87 (3H, s, COOMe), 3.78 and 3.87 (3H, COOMe), 3.67 (3H, s, OCH_3), 2.79 (2H, H-21′), 2.06 and 2.09 (3H, OCOMe), 1.39 (4H, H-19, H-19′), 0.94 (3H, H-18), 0.91 (3H, H-18′); VCR·SO_4 ^{1}H NMR (D_2O, 360 MHz) δ 8.95 (1H, s, N−CHO), 7.66 (1H, d, J = 7.56 Hz, H-9′) 7.41 (1H, d, J = 7.49 Hz, H-12′), 7.27 (1H, s, H-9), 7.27 (1H, t, H-10′), 7.20 (1H, t, H-11′), 7.08 (1H, s, H-9), 6.02 (1H, m, H-14), 5.78 (1H, d, J = 10.37 Hz, H-15), 5.16 (1H, H-17), 4.74 (1H, s, H-2), 3.95 (3H, s, COOMe), 3.74 (3H, s, COOMe), 3.71 (3H, s, ArOMe), 3.28 (2H, s, H-21′), 2.10 (3H, s, OCOMe), 1.56 (2H, q, H-19), 1.42 (2H, q, H-19′), 0.95 (3H, t, J = 7.4 Hz, H-18), 0.83 (3H, t, J = 6.85 Hz, H-18′); ^{13}C NMR ($CDCl_3$ 90 MHz) δ 173.91 (C-16′-CO), 170.22 and 169.85 (COO), 170.03 and 169.95 (OCO), 160.62 and 160.16 (NCHO), 157.61 and 157.17 (C-11), 140.77 and 140.65 (C-13), 134.88 (C-13′), 129.97 (C-2′), 129.81 (C-8′), 129.33 (C-15), 127.79 (C-10), 124.58 (C-8), 124.47 (C-14), 123.62 (C-10′), 122.20 (C-9), 118.69 (C-11′), 118.31 (C9′), 117.65 (C-7′), 110.41 (C-12′), 101.60 and 94.91 (C-12), 81.03 and 79.31 (C-16), 75.30 and 73.69 (H-17), 71.85 (C-2), 68.94 (C-20′), 64.27 and 64.47 (C-21), 63.70 (C-21′), 55.78 (Ar−

OMe), 55.62 (C-5′), 52.67 (C-7), 52.53 (C-16′), 52.43 (16′-OMe), 52.27 (OMe), 49.43 (C-3), 48.79 (C-3′), 47.20 (C-5), 42.13 (C-20 and C-15′)), 40.91 (C-6), 34.15 (C-17′), 33.9 (C-19′), 30.30 (C-19 and C-6′), 29.53 (C-14′), 20.20 (CO*Me*), 7.98 (C-18), 6.55 (C-18′).

Desacetylvincristine[18] (**4**): ^{1}H NMR ($CDCl_3$, 300 MHz); ^{13}C NMR ($CDCl_3$ 78 MHz).

HRP (Type VI, expressed activity of 260 purpurogallin units/mg, Lot 69F-9800), the copper oxidase, ceruloplasmin (type X, expressed oxidase activity of 4698 units/ml), and hydrogen peroxide were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade in quality.

Peroxidative Metabolism of VCR (1). Analytical-scale incubations of VCR·SO_4 with HRP and H_2O_2 were conducted in 50-mL DeLong culture flasks each containing 10 mL of 0.1 M, pH 7.9 sodium phosphate buffer, HRP (2 mg, 520 purpurogallin units), 2.2 mM H_2O_2 (1 mL), and VCR·SO_4 (2 mg, 2.2 μmol). Mixtures were incubated on a NBS G24 gyrotory shaker at 250 rpm and 37 °C. Samples of 2 mL were taken at 30 and 120 min, adjusted to pH 10 with 3 N NH_4OH, and extracted with equal volumes of EtOAc by vortex-mixing and centrifuging at 2800 rpm for 3 min. The solvent layer was evaporated under N_2, and the dry sample was dissolved in 150 μL of EtOAc followed by spotting 18 μL of the solution onto a Si gel GF_{254} TLC plate. Under these conditions VCR was transformed to one major product **5** at R_f 0.6 in estimated (TLC) yields of 70 and 90%, at 30 and 120 min, respectively. Control experiments included reactions without HRP, H_2O_2, or VCR·SO_4. Results from control experiments indicated no significant product formation.

Preparative Oxidation of VCR (1) with HRP. A total of 80 mg of VCR·SO_4 (87 μmol) was added to a mixture containing 600 mL of 0.1 M, pH 7.9 potassium phosphate buffer, 2.2 mM H_2O_2 (40 mL, final reaction concentration 0.14 mM), and HRP (20 mg, 5200 purpurogallin units). The reaction mixture was divided evenly among 40 125-mL DeLong culture flasks and incubated on a shaker at 250 rpm and 37 °C for 3 h. TLC monitoring of the reaction as before indicated that a relatively nonpolar metabolite (R_f 0.6) was formed in an estimated 90% yield.

The reaction mixtures were combined, made alkaline to pH 10 with 3 N NH_4OH and extracted three times with equal volumes of EtOAc. The EtOAc extract was dehydrated over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure to give 73 mg of brown residue. This residue was applied onto two, 1-mm Si gel GF_{254} preparative TLC plates and developed with $CHCl_3$−MeOH (10:1, v/v). The major transformation product band at R_f 0.6 was scraped from the plates, combined, and extracted with Me_2CO (3 × 2 mL) by vortex-mixing and performing centrifugation at 2800 rpm for 2 min. The extract was filtered through Me_2CO-washed glass wool, and the filtrate was evaporated under N_2. The product was concentrated to dryness in an Abderhalden vacuum oven to give 27 mg of pure metabolite (**5**, 37%): HRMS *m*/*z* (M + 1) 839.3881 (calcd for $C_{46}H_{55}N_4O_{11}$, 839.3872); ^{1}H NMR ($CDCl_3$ 600 MHz) δ 8.81 and (1H, s, NCHO), 7.93 (1H, s, NH), 7.82 (1H, d, J = 5.8 Hz, H-21′), 7.49 (1H, d, J = 7.56 Hz, H-9′), 7.78 and 6.92 (1H, s, H-12), 7.13 and 7.18 (3H, m, H-10′, H-11′, H-12′), 6.86 and 6.78 (1H, s, H-9), 5.94 (1H, m, H-14), 5.43 (1H, d, J = 10.12 Hz, H-15), 5.27 and 5.23 (1H, s, H-17), 4.77 and 4.54 (1H, s, H-2), 3.86 (3H, s, COOMe), 3.78 (3H, s, COOMe), 3.73 (3H, s, ArOMe), 2.07 (3H, s, OCOMe), 1.26 (2H, H-19); ^{13}C NMR ($CDCl_3$ 90 MHz) δ 210.96 and 211.28 (C-20′), 174.48 (C-16′), 171.65 and 171.48 (COO), 171.31 (OCO), 164.47 (C-21′), 161.72 and 161.04 (N−CHO), 158.80 and 158.10 (C-11), 142.35 (C-13), 136.34 (C-13′), 130.39 (C-15), 130.38 (C-2′), 126.63 (C-10), 125.80 (C-8′), 125.70 (C9 and C-14), 123.45 (C-8, C-10′), 120.23 (C-11′), 118.45 (C9′), 111.80 (C-7′ and C-12′), 102.22 and 95.27 (C-12), 82.0 and 80.26 (C-16), 76.39 and 74.67 (C-17), 73.92 and 72.88 (C-2), 65.05 (C-21), 56.34 (ArOMe and C-5′), 53.39 (C-7), 53.16 (C-16′, C-16′-OMe), 52.87 (OMe), 50.24 (C-15′), 49.85 (C-3, C-5, C-3′), 42.63 (C-20), 42.53 (C-6), 37.49 (C-19′), 35.63 (C-17′), 30.80 (C-14′, C-19), 25.38 (C-6′), 21.20 (CO*Me*), 8.0 and 7.86 (C-18), 7.8 and 7.3 (C-18′).

Oxidation of VCR (1) with Ceruloplasmin. Analytical-scale incubations of VCR·SO_4 with ceruloplasmin-CPZ were carried out in 50-mL DeLong culture flasks each containing 10 mL of 0.2 M, pH 5.5 NaAc buffer, CPZ·HCl (1 mg, 2.9 μmol) dissolved in 10 mL of MeOH, ceruloplasmin (76.4 units, 20 mL), and 1 mg (1.1 μmol) of VCR·SO_4 dissolved in 50 mL of the above buffer. The mixtures were incubated on a shaker at 37 °C and 250 rpm, and the reactions were monitored by TLC.

A sample of the reaction mixture (1 mL) was withdrawn after 60 min, adjusted to pH 9 with 3 N NH_4OH, and extracted with equal volumes of CH_2Cl_2. The solvent layer was dried under a N_2 stream, 150 μL of CH_2Cl_2 was added, and 15 μL of the solvent was spotted onto TLC plates for analysis. The metabolite (R_f 0.6) formed in more than 50% yield by TLC estimation. No significant amounts of product were detected in controls that consisted of reaction mixtures of VCR without ceruloplasmin, CPZ, or both ceruloplasmin and CPZ.

Preparative Oxidation of VCR (1) with Ceruloplasmin. A total of 81 mg (87 μmol) of VCR·SO_4 dissolved in 9 mL of H_2O was added to a mixture containing 720 mL of 0.2 M, pH 8.8 NaAc, 81 mg of CPZ·HCl, (0.24 mmoles) dissolved in 9 mL of MeOH, and ceruloplasmin (6761 units, 1.44 mL). Reaction mixtures were divided evenly among 36 128-mL DeLong culture flasks and incubated at 37 °C and 280 rpm for 2 h on a New Brunswick G-25 gyrotory shaker. TLC indicated the presence of a major metabolite at R_f 0.6 in incubation sample extracts.

Enzyme reaction mixtures were combined, adjusted to pH 9 with NH_4OH, and extracted four times each with 800 mL of CH_2Cl_2. The CH_2Cl_2 extract was dehydrated over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure to give 177 mg of dark brown residue.

Half of the residue (88 mg) was dissolved in 250 mL of C_6H_6−MeOH (10:1), loaded onto a 14-g Si gel column (1.1 cm × 19 cm), and eluted with the same solvent at a flow rate of 0.15 mL per min. Fractions from 29 to 64 were combined to give 27 mg of crude product, which was further purified by preparative TLC ($CHCl_3$−MeOH, 10:1, v/v). The metabolite band at R_f 0.6 was scraped from the TLC plate and extracted three times each with 2 mL of Me_2CO by vortex-mixing and centrifuging at 2800 rpm for 2 min. The extract was filtered

and the filtrate evaporated under N_2 and vacuum-dried to give 7 mg of light yellow metabolite **5**.

The remaining 89 mg of extract from the preparative-scale reaction was directly purified by preparative TLC to give an additional 6 mg of **5** for a total of 13 mg (18% yield).

CEM-CCRF Leukemic Cell System Bioassay. The metabolite **5** was evaluated for biological activity using a range of concentrations in the in vitro CEM-CCRF leukemic cell system using the method described by Foley[25] and Grindey.[26] IC_{50} (50% inhibitory concentrations) values (±10%) were obtained by using triplicate samples at three different drug concentrations, and mean values are shown in Table 1.

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