In order to observe epoxidase activity *only*, oxidosqualene cyclase inhibitor, *N*,*N*-dimethyldodecylamine *N*-oxide,[21] was added (10 μL of a 24 mM solution in 2-propanol, final concentration = 100 μM).

Oxidosqualene Cyclase Plus Squalene Epoxidase. The enzyme preparation was identical with that of squalene epoxidase, except that no *N*,*N*-dimethyldodecylamine *N*-oxide was added.

Inhibition of SE and OSC: IC_{50} Determinations. Test tubes containing either squalene epoxidase or oxidosqualene cyclase enzyme solutions (240 μL each, as described above) were warmed to 37 °C. After 10 min, squalene analogues were added (1 μL in 2-propanol) to give final inhibitor concentrations of 0, 4, 20, 40, 200, and 400 μM. After an additional 10 min, [^{14}C]-squalene (2 μL in 2-propanol, approximately 20 000 dpm, 33 μM) was added to each enzyme solution. Incubation was continued for another 50 min and then stopped by the addition of 10% KOH–methanol (240 μL). After 1 h at 37 °C, each mixture was extracted with CH_2Cl_2 (1 mL each); the resulting organic extracts were dried ($MgSO_4$) and redissolved in a small amount of CH_2Cl_2 (100 μL), and the triterpene components were separated by TLC. Radiochemical analysis, using either linear analysis or scintillation counting, showed conversion of squalene to either 2,3-oxidosqualene (for the epoxidase assays) or a mixture of lanosterol and 2,3-oxidosqualene (for the cyclase assays). Inhibitor-free assays showed approximately 30% conversion to product, and radiochemical recoveries of 80–90% were routinely achieved.

Inhibition of SE and OSC: Kinetic Analysis. Squalene epoxidase solutions were warmed to 37 °C. After 10 min, inhibitors were added to give three sets of inhibitor concentrations: 0 mM, the IC_{30} value, and the IC_{50} value. After a 10-min preincubation, [^{14}C]squalene was added to give final substrate concentrations of 13, 17, 25, and 40 μM for each set of inhibitor concentrations. The enzyme solutions were incubated for 50 min and then quenched by the addition of 10% KOH–methanol and the products were isolated as described above.

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Supplementary Material Available: Figures representing a Lineweaver–Burk plot of **4** and **12**, and minimization results for the modified isopropylidene region of **1–16**, and superimpositions of each analogue with squalene (12 pages). Ordering information is given on any current masthead page.

In Vitro Metabolic Transformations of Vinblastine: Oxidations Catalyzed by Human Ceruloplasmin

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The dimeric *Vinca* alkaloid vinblastine (VLB) undergoes metabolic transformation to three products in a reaction catalyzed by the human serum copper oxidase ceruloplasmin. The enzyme reaction requires chlorpromazine as a shuttle oxidant, and the course of the oxidation reaction appears to be subject to the nature of the shuttle oxidant used. Preparative-scale incubations have resulted in the isolation of three products, which were characterized by chemical and spectral analyses. The metabolites were identified as the ring fission product catharinine, obtained by oxidation of the *Iboga* ring system; an enamine/ether derivative obtained by oxidation of the *Aspidosperma* portion of VLB; and a metabolite embodying the same structural changes in both parts of the vinblastine dimeric structure. Catharinine is identical with the product of VLB oxidation obtained by peroxidase oxidation. The other two products are new metabolites and are derivatives of VLB. All of the metabolites are less active than VLB when tested in vitro vs the human T-cell leukemic cell line (CRFF-CEM).

The *Vinca* alkaloids vinblastine (VLB) (1) and vincristine (VCR) are widely used in cancer chemotherapy.[1] Although several studies have shown that VLB (1) is extensively metabolized in mammals,[2-12] little is known about the metabolites or their possible role in either the mechanism of action or the dose-limiting toxicities. Our investigations have focused on elaborating biochemical and chemical mechanisms by which dimeric and monomeric *Vinca* alkaloids are oxidized by different types of enzymes. The susceptibility of *Vinca* alkaloids to one-electron oxidation reactions is well documented in our laboratories.[13,14] Catalysis by mouse liver microsomes, microorganisms, peroxidases, and copper oxidases of the monomeric *Vinca* alkaloid vindoline yielded several metabolites, all of which were isolated and fully characterized.[13,15-18] Their identification and probes of the mechanism by which they are formed provide a clear basis for understanding metabolic transformations of more complex *Vinca* alkaloid dimers.

Ceruloplasmin is a member of the blue copper oxidases which also include laccases and ascorbate oxidase.[19] Copper oxidases occur widely among mammals, plants, and microorganisms. The enzymes are important electron-transfer agents in biological systems, and they catalyze oxidation reactions by the direct removal of substrate

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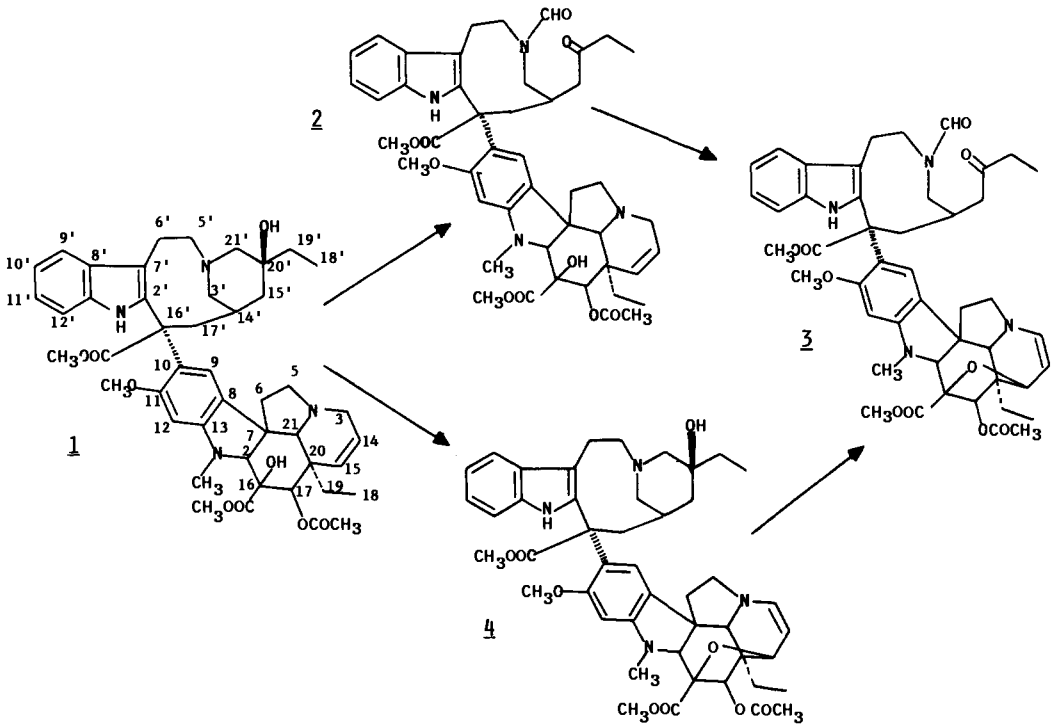

Figure 1. The structures of vinblastine (1), catharinine (2), and metabolites 3 and 4.

electrons with the concomitant reduction of enzyme-bound Cu^{2+} to Cu^{+}, followed by the transfer of electrons to molecular oxygen.[20-23]

We recently reported the peroxidase-catalyzed oxidation of VLB (1) to form the metabolite catharinine (2)[24] (see Figure 1). As a part of ongoing, in vitro metabolic studies, we examined the oxidation of the clinically important VLB (1) by human ceruloplasmin. VLB (1) is not a true substrate for the copper oxidase. Rather, the alkaloid is oxidized only in the presence of a "cofactor" or shuttle oxidant as seen with other xenobiotics.[25] With chlorpromazine as shuttle oxidant, VLB (1) was oxidized to several products which were isolated and identified.

The isolation, structure characterization, and the biological evaluation of these oxidation products as well as a proposed pathway for their formation are described herein.

Results and Discussion

Although VLB (1) has been in clinical use for nearly 30 years, the only metabolites identified so far are desacetylvinblastine from metabolic studies on rats[3,12] and catharinine (2) obtained by in vitro oxidation of VLB (1) with peroxidase.[24] Metabolic alterations in the structures of Vinca alkaloid dimers may result in their bioactivation, bioinactivation, or increased neurotoxicity. It is important, therefore, to identify metabolites in order to understand the biochemical mechanisms by which they are formed, and to establish their biological activities.

No products were obtained from incubations of VLB (1) with human serum ceruloplasmin unless a "cofactor",[6] or shuttle oxidant,[25] was present in incubation mixtures. Many so-called "cofactors" such as serotonin, chlorpromazine, (dimethylamino)phenylenediamine, and *p*-phenylenediamine are true substrates for copper oxidases. They are oxidized to chemically reactive radical or radical cation species that are capable of catalyzing further chemical reactions with "pseudosubstrates" like VLB (1). Ceruloplasmin oxidation reactions were greatly enhanced when chlorpromazine was used as a shuttle oxidant, as evidenced by prominent increases in oxygen consumption in analytical oxygen electrode experiments. Since other shuttle oxidants failed to enhance VLB oxidations, chlorpromazine was used in all further analytical and preparative-scale incubations. The relevance of shuttle oxidant mediated oxidations of compounds like VLB (1) becomes more important since this alkaloid is used only in combination chemotherapy for the treatment of various types of cancer. One agent that is used in combination with VLB (1) is etoposide. Although etoposide undergoes one-electron oxidation,[26] it was not a shuttle oxidant for VLB oxidation. However, a 30% decrease in O_2 con-

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sumption was measured when etoposide was added to complete incubation mixtures containing VLB (1) and chlorpromazine. Chromatographic evaluation of incubation mixtures containing both VLB (1) and etoposide revealed a mixture of unidentified alkaloid products different than that obtained with VLB (1) alone. This suggests the possibility that the course of copper oxidase mediated oxidations of VLB (1), and indeed those catalyzed by other enzyme systems, may be changed in the presence of etoposide.

Preparative-scale incubation of VLB (1) with ceruloplasmin resulted in the production of three metabolic products which were isolated and characterized as 2–4. Compound 4 was barely detectable in analytical-scale incubation mixtures. Metabolite 2 was found to be identical (HPLC, TLC, mass spectrum, and ^{1}H NMR) with catharinine, which was previously isolated from horseradish peroxidase incubation mixtures and characterized in our laboratories.[24]

Metabolite 3, the major copper oxidase product, exhibited a molecular ion peak at m/z 823.3878 (M + 1) consistent with molecular formula $C_{46}H_{55}N_4O_{10}$ (calculated 823.3918). Comparison of the proton NMR spectrum of 3 with that of VLB (1) indicated structural differences in both the *Iboga* and *Aspidosperma* parts of the molecule. An *N*-formyl proton signal in the *Iboga* portion (singlet at 8.10 ppm) was clearly evident, and suggestive of an *Iboga* structure similar to 2.[24] The *Aspidosperma* portion of the molecule exhibited notable differences, especially in the olefinic region of the ^{1}H NMR spectrum. Signals at 5.31 (d, H-15) and 5.86 ppm (dd, H-14) in VLB (1) were replaced by new signals at 4.90 (dd, H-14) and 5.90 ppm (d, H-3). These signals suggested the presence of an ether linkage between C-16 and C-15 as shown in 3. This was further supported by the ^{13}C NMR spectrum where the triplet signal for C-3 (50.0 ppm in 1) was replaced by a doublet (139.2 ppm in 3). The significant chemical shift changes for C-14 and C-15 from 124.3 and 129.7 ppm to 97.5 and 77.2 ppm, respectively, also support a structure of an enamine with an ether linkage. All other ^{13}C NMR chemical shift assignments (Table I), which were confirmed on the basis of a ^{1}H–^{13}C shift-correlated 2D NMR experiment, were in complete agreement with the structure of 3. Similar resonances have been reported for the natural product craspidospermine which contains a similar ether linkage/enamine moiety.[27] Additional evidence in support of the metabolite structure as shown in 3 came from a COSY (homonuclear correlated spectroscopy) experiment where distinct cross peaks correlating H-14 (dd, J = 6.2, 6.3 Hz) to both H-3 (d, J = 6.3 Hz) and H-15 (d, J = 6.2 Hz) were clearly evident in the spectrum of metabolite 3.

Metabolite 4 was highly unstable and decomposed upon standing. However, it was possible to obtain a sample that was about 90% pure for proton and carbon NMR, and mass spectral data supported its structure. The high-resolution mass spectrum of 4 showed a molecular ion at 809.4172 (M + 1), consistent with a molecular formula of $C_{46}H_{57}N_4O_9$ (calculated 809.4126). As for 3, the ^{1}H NMR for the *Aspidosperma* portion of 4 also exhibited new signals at 4.20 (d, H-15), 4.92 (dd, H-14), and 5.95 ppm (d, H-3). These signals, plus ^{13}C NMR spectral properties, indicated identical structures for the *Aspidosperma* portion of both 3 and 4. Unlike 3, however, the carbon signals for the *Iboga* half of 4 remained unchanged from those in VLB (1). The remaining proton and carbon resonances were in total agreement with structure 4.

Table I. ^{13}C NMR Spectral Properties of VLB (1) and Metabolites 2 and 3[a]

carbon	VLB (1)	2	3
2	83.0	83.63	84.21
3	50.0	50.01	139.20
5	50.0	50.45	49.17
6	44.3	43.87	53.22
7	52.9	53.30	50.75
8	122.6	123.59	128.91
9	123.1	124.93	123.86
10	120.4	120.12	118.73
11	157.8	157.92	157.89
12	93.9	93.68	92.31
13	152.5	153.05	150.45
14	124.3	124.58	97.55
15	129.7	129.88	77.20
16	79.3	79.69	86.23
17	76.1	76.51	75.14
18	8.1	8.40	9.00
19	30.4	30.83	22.29
20	42.3	42.72	47.23
21	65.2	66.13	64.19
COO	170.6	170.89	169.69
OMe	51.8	52.19	52.16
OCO	171.4	171.72	169.89
AcMe	20.7	21.11	20.90
ArOMe	55.3	55.52	55.53
NMe	38.0	38.31	36.28
2′	130.9	132.71	132.77
3′	47.5	49.46	49.46
5′	55.5	55.73	49.64
6′	28.7	25.28	25.19
7′	115.9	111.53	111.61
8′	129.0	128.37	128.86
9′	118.1	117.68	117.52
10′	122.2	122.50	122.53
11′	118.8	119.31	119.26
12′	110.2	110.78	110.90
13′	134.7	135.29	135.32
14′	29.2	29.59	29.95
15′	40.0	51.09	51.11
16′	55.3	55.52	55.96
17′	34.1	34.88	35.26
18′	6.7	7.89	7.77
19′	34.1	37.44	37.89
20′	68.6	210.38	210.17
21′	63.1	163.45	163.51
CO	174.6	174.19	174.13
OMe	52.0	52.35	52.21

[a] Assignments were confirmed by ^{1}H–^{13}C shift-correlated 2D NMR spectroscopy, and multiplicities were determined according to a DEPT experiment.

The formation of metabolite 3 can be explained by a combination of enzymic/chemical transformations as shown in Scheme I. This scheme is based on extensive previous work which clearly demonstrated the involvement of iminium and enamine intermediates,[13,15,16] carbinolamines,[25] and ring fission[14,24] reactions in enzymic oxidations of several *Vinca* alkaloids. The pathway for the formation of 3 is probably similar to that observed in the oxidative ring fission of VLB (1) by peroxidase.[24] With the copper oxidase/chlorpromazine system the initial oxidation step in the *Iboga* ring system most probably involves intersystem oxidation of VLB (1) by chlorpromazine cation radical[16] to yield 5. Evidence for the existence of chlorpromazine cation radical comes from the appearance of a red color in complete reaction mixtures.[16] Formation of iminium 6 and addition of water would give the carbinolamine diol 7 for subsequent periodate-like diol cleavage to form 2. The copper oxidase mediated cleavage of the putative diol intermediate represents still another example of a metalloenzyme-catalyzed oxidation of vicinal diols, similar to that observed for VLB (1) with per-

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Scheme I. Conversion of Vinblastine (1) to Metabolites 2–4

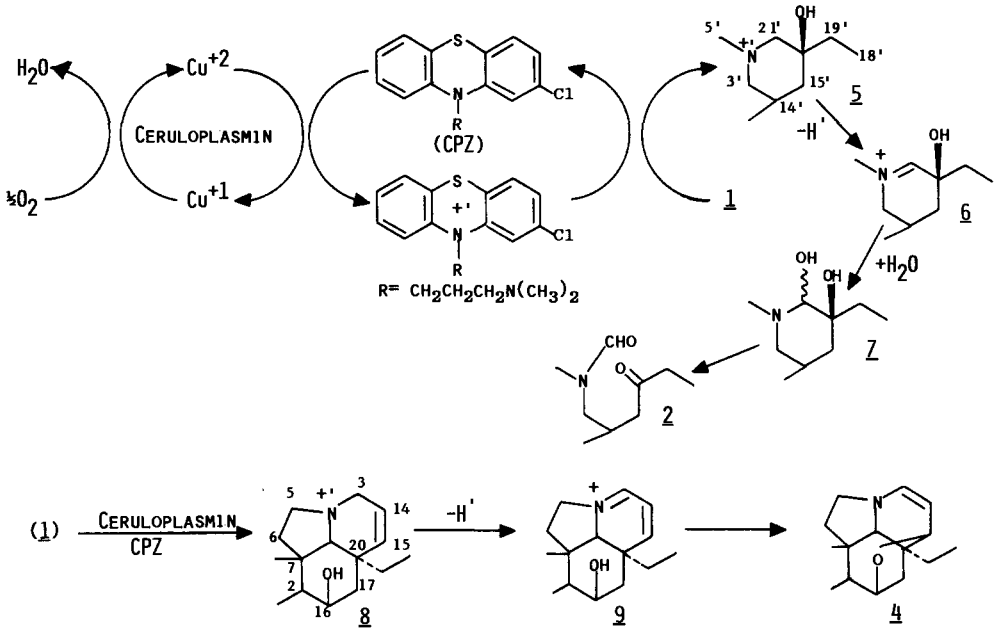

Table II. IC_{50} Values of Vinblastine, Some Dimeric *Vinca* Alkaloids, and Metabolites 2 and 3 vs the CRFF-CEM T-Cell Leukemic Cell Line

compd tested	IC_{50}, μg/mL	compd tested	IC_{50}, μg/mL
vinblastine (1)	0.1	vinleurosine	0.4
vincristine	0.025	metabolite 2	7.7
vinrosidine	1.0	metabolite 3	14.0

oxidase[24] and cholesterol side-chain degradation by cytochrome P-450.[28]

Enamine 4 likely forms by intramolecular nucleophilic 1,4-addition of the 16-hydroxyl group of VLB to position 15 of reactive iminium 9, in a process similar to the well-established oxidation pathway for vindoline.[13,15,16] It is difficult to determine whether the pathway for formation of 3 proceeds through 2 or 4 as the first oxidation step. However, 2 and 4 yield 3 when incubated with the ceruloplasmin/chlorpromazine system, thus confirming their intermediacy in the oxidation process.

Compounds 2 and 3 were examined for biological activity in the *Vinca* alkaloid sensitive CRFF-CEM human leukemic test system in vitro.[29,30] As shown in Table II, the IC_{50} values for 2 and 3 were 77 and 140 times more than that for VLB (1). In general, these results follow previous structure–activity relationship patterns of the *Vinca* alkaloids in which alterations made in the *Iboga* half of the dimeric alkaloid structure reduce oncolytic activity.[31]

Experimental Section

Vinblastine sulfate was kindly provided by OMNICHEM, PRB, Belgium, or Eli Lilly and Co. and possessed physical and spectral properties consistent with those reported in the literature.[24,32] Human serum ceruloplasmin (type X, expressed oxidase activity of 3080 units/mg) and chlorpromazine were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals used were reagent grade and were supplied from commercial sources.

Low-resolution mass spectra were obtained by using an EI NERMAG R 10-10 C (70-eV ionization potential). High-resolution mass spectra were recorded on ZAB-HF mass spectrometer. ^1H and ^{13}C NMR spectra were recorded in deuterated chloroform on a Bruker WH-360 FT spectrometer operating at 360.134 (^1H) and 90.556 (^{13}C) MHz, respectively. The chemical shift values are reported in ppm. Standard pulse sequences were used for COSY[33] and HETCOR[34] experiments. IR spectra were performed in chloroform solution (0.1-mm NaCl cells) or KBr pellets by using a Nicolet 5DXB FT-IR spectrophotomer. UV spectra and enzyme assays were determined by using a Shimadzu UV-160 spectrophotometer equipped with a thermostated cell holder.

Chromatography. Thin-layer chromatographic analyses (TLC) were carried out on 0.25 mm thick silica gel GF_{254} plates (Merck). The solvent system used was chloroform/methanol (95:5 v:v) in which the R_f values of VLB (1) and metabolites (2–4) were 0.32, 0.52, 0.41, and 0.37, respectively. Spots were visualized by UV fluorescence quenching at 254 nm and by spraying the developed plates with ceric ammonium sulfate (CAS) or Dragendorff's reagent. The adsorbent used for column chromatography was silica gel (60–200 mesh, Baker 3405) that was oven-activated at 110 °C for 30 min prior to use.

High-performance liquid chromatography (HPLC) was performed by using a Waters Associates (Milford, MA) Model 6000A pump, a U6K Universal injector, and an ISCO V^4 variable-wavelength UV detector. Samples were analyzed on an Alltech (Deerfield, IL) Versapack C-18 column (25 cm × 4.6 mm, 10 μ) protected with a guard column filled with the same material. The mobile phase consisted of acetonitrile/0.005 M dibasic ammonium phosphate and 0.005 M 1-pentanesulfonic acid/H_2O (7:3 v:v). Concentrations are expressed relative to the volume of water. The flow rate was 1 mL/min, and absorbance was monitored at 220 nm. With vincristine sulfate as an internal standard, the retention volumes of vincristine, vinblastine, 2, 3, and 4 were 10, 14, 5, 8, and 9 mL, respectively.

Enzyme Incubation Procedures. Analytical-scale incubations of VLB (1) with the ceruloplasmin–chlorpromazine system were carried out in 50-mL Delong flasks each containing 7 mL of buffer (0.2 M sodium acetate, pH = 5.50), 2 mg (6.29 μmol) of chlorpromazine in 0.1 mL of MeOH, 50 μL of ceruloplasmin (128 units), and 2 mg (2.47 μmol) of vinblastine sulfate in 0.1 mL

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of MeOH. The mixtures were incubated on a New Brunswick Scientific Co. G-24 gyrotory shaker (250 rpm) at 37 °C, and reactions were monitored by TLC. Samples were withdrawn at various time intervals, adjusted to pH 10 with ammonium hydroxide solution and extracted with equal volumes of ethyl acetate. Incubation extracts (30 μL) were spotted onto TLC plates for analysis. Under these conditions VLB was completely consumed in the reaction at 3 h, yielding three metabolites by TLC. Spots of chlorpromazine (R_f = 0.27) and chlorpromazine oxidation product (R_f = 0.09) were also detected. No metabolites were produced in controls which consisted of incubation mixtures with substrate plus all reaction components except enzyme.

Oxidation Rate of VLB (1) with Human Ceruloplasmin. The rate of the enzyme oxidation reaction was determined by using an oxygen electrode as previously described.[16] Incubations were conducted as above by using ceruloplasmin (54 units), VLB (1) (1.0 mg, 1.23 μmol), and chlorpromazine (9.4 mg, 2.65 μmol). Oxygen uptake was measured by a recorder which was calibrated to measure full-scale deflection for 0.83 μmol of oxygen. Under these conditions, VLB (1) was oxidized at a rate of 9.23 μmol of oxygen/(L·min).

Preparative-Scale Oxidation of VLB with Human Ceruloplasmin. A total of 160 mg (0.198 mmol) of VLB sulfate dissolved in 8 mL of H_2O was added to a mixture containing sodium acetate buffer (0.2 M, pH = 5.51, 562 mL), chlorpromazine (160 mg, 0.503 mmol) dissolved in 8 mL of methanol, and human ceruloplasmin (4644 units, 1.8 mL). Twenty-five-milliliter volumes of the incubation mixture were divided evenly into 125-mL Delong culture flasks and incubated at 37 °C for 3 h on a New Brunswick gyrotory shaker (250 rpm). Enzyme reaction mixtures were combined, alkalinized to pH 10 with ammonium hydroxide solution, and extracted 4 times with ethyl acetate (600 mL, 3 × 300 mL). The ethyl acetate extract was dehydrated over anhydrous sodium sulfate and evaporated under reduced pressure to give 292 mg of a dark brown residue. The residue (290 mg) was purified over a silica gel column (30 × 2 cm, 29 g) packed as a slurry in chloroform. Elution began with 1% methanol in chloroform while 7-mL fractions were collected. Fractions 10–13 contained metabolite (2) (3 mg, 2%). Metabolite 2 was found to be identical [TLC, HPLC, mass spectrum (CI, m/z 824), and ^{1}H NMR] with catharinine, which was previously isolated and characterized in our laboratory.[24] Fractions 14–20 contained a pure (by TLC) single metabolite (3) (22 mg, 14%). Fractions 21–33 contained a mixture of metabolite 3 and metabolite 4 (77 mg). Further purification of this mixture by column chromatography resulted in the isolation of metabolite 4 as a highly unstable material (17 mg, 11%). Traces of the starting material (VLB, 1), unreacted chlorpromazine, and a chlorpromazine oxidation product were also recovered from the column. The total recovery of VLB products was 82%, the remainder most likely lost during chromatography. Metabolites exhibited the following physical and spectral properties.

Metabolite 3. HRMS, 823.3878 (M + 1), calculated 823.3918 for $C_{46}H_{55}N_4O_{10}$; IR ($CHCl_3$); UV (MeOH) λ_{max} 215 nm (log ε 4.58), 274 nm (log ε 4.03); ^{1}H NMR ($CDCl_3$) (inferred from COSY experiment) 0.85 (3 H, t, J = 7.2 Hz, Me-18′), 1.00 (3 H, t, J = 7.5 Hz, Me-18), 2.70 (1 H, m, H-19′), 2.80 (1 H, m, H-19′), 4.24 (1 H, d, J = 6.2 Hz, H-15), 4.90 (1 H, dd, J = 6.2, 6.3 Hz, H-14), 5.50 (1 H, s, H-17), 5.90 (1 H, d, J = 6.3 Hz, H-3), 6.80–7.09 (1 H, m, H-9, H-12), 7.10 (3 H, m, H-10′, H-11′, H-12′), 7.45 (1 H, dd, J = 7.0, 1.2 Hz, H-9′), 8.10 ppm (1 H, s, CHO); ^{13}C NMR (Table I).

Metabolite 4. HRMS, 809.4172 (M + 1), calculated 809.4126 for $C_{46}H_{57}N_4O_9$. NMR properties are given under Results and Discussion.

Incubation of 2 and 4 with Ceruloplasmin. Metabolites 2 and 4 were incubated separately (1.5 mg, 1.86 μmol in 0.1 mL of MeOH) in reaction mixtures containing sodium acetate (7 mL, 0.2 M, pH = 5.51), chlorpromazine (1.5 mg, 4.72 μmol in 0.1 mL of MeOH), and 30 μL ceruloplasmin (85 units). The reaction mixtures were incubated at 37 °C on a shaker at 250 rpm for 3 h before being alkalinized with ammonium hydroxide solution to pH = 10 and extracted with equal volumes of ethyl acetate. The ethyl acetate solutions were dehydrated over anhydrous sodium sulfate and evaporated to dryness. TLC and HPLC analyses clearly indicated that both substrates were converted to 3. No attempts were made to purify 3 from the extracts.

CRFF-CEM Leukemia Cell System Bioassay. Metabolites were subjected to bioassay using a range of doses in the CRFF-CEM (in vitro) leukemic cell system as described by Foley[29] and Grindey.[30] IC_{50} (50% inhibitory concentrations) values (±10%) were obtained by using triplicate samples at three different drug concentrations, and mean values are presented in Table II.

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