assessed correlations from the pK_a* values in mixed solvents are in close agreement with each other and with the value of UDCA. All the measurements have been carried out at 25 ± 0.01 °C.

Hepatic Uptake. Isolated liver perfusions were performed as described by Mortimore.[25] The perfusate consisted of Krebs–Ringer bicarbonate, pH 7.4, containing glucose (100 mg/mL) and bovine serum albumin (3% w/v) (fraction V essentially fatty acid free, Sigma Chemical Co., St. Louis MO). Evans blue (1 mg/g liver) was added as marker of the flow rate and recovery. The outflow was collected at 10-s intervals immediately after injection of a 10-μmol/L solution of CUDCA (200 μL). In all experiments, CUDCA (**5**) and UDCA were randomly injected. The concentrations of BA in the outflow sample were measured enzymatically.[23] The Evans blue was directly evaluated in each sample in a Perkin-Elmer spectrophotometer at 610 nm. Albumin concentration in the perfusate was measured by the method of Lowry et al.[26] The percentage of outflow was corrected for the Evans blue, and the liver uptake was calculated as 100% − outflow.

Bile Lipid Secretion. Sprague–Dawley male rats (300–330 g) were used. The rats were anesthetized with ethyl carbamate, and the bile was collected. The bile acids, CUDCA and UDCA, were administered intravenously as sodium salts through the femoral vein at a dose of 50 mg per animal (five rats each group). Bile samples were collected at 15-min intervals for a 1-h period. The concentrations of bile acids, cholesterol, and phospholipids in bile samples were determined by enzymatic procedures.[23,27,28] The bile acid compositions were also determined by an HPLC method[29] and by TLC on silica gel G plates, 250-μm thickness. The conjugated bile acids (glycine/taurine) were separated from the corresponding unconjugated BA by the solvent system propionic acid/isoamyl acetate/H_2O/1-propanol (75:100:25:5, v/v). Biliary lipid secretion, calculated from the volume of the excreted bile and from the biliary lipid concentration, was expressed as micromoles per kilogram per hour.

The results of the physicochemical and biological evaluations of **5a** clearly indicate that the cyclopropane ring at C-22 and C-23 exerts an effect that could be explained by the unusual electronic and steric characteristics of the three-membered ring. The new bile acid presents promising properties as a candidate drug both for cholesterol gallstones dissolution and for improving the bile flow and the biliary lipid secretion. The major new features are as follows: (1) Optimum critical micellar concentration: this means that the "coupling" with cholesterol and phospholipid is favorable to the formation of a micellar solution. As a consequence, more cholesterol will be solubilized by this BA. (2) CUDCA is able to inhibit selectively the cholesterol secretion; consequently, the bile secreted during feeding of this BA is less saturated in cholesterol. In addition, CUDCA largely increases the bile flow, and during this effect the bile is still enriched in BA and phospholipids and less in cholesterol.

More detailed biochemical studies devoted to explaining the metabolism in terms of deconjugation and dehydroxylation are in progress.

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Registry No. **1b**, 128-13-2; **1c**, 6533-77-3; **2**, 89414-89-1; **3** (22*R*,23*R*), 89414-90-4; **3** (22*R*,23*S*), 89495-32-9; **3** (22*S*,23*R*), 89495-33-0; **3** (22*S*,23*S*), 89495-34-1; **5** (22*R*,23*R*), 89414-91-5; **5** (22*R*,23*S*), 89495-35-2; **5** (22*S*,23*S*), 89495-36-3; **5** (22*S*,23*R*), 89496-31-1; N_2CHCO_2Et, 623-73-4.

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Formation of a Reactive Iminium Derivative by Enzymatic and Chemical Oxidations of 16-*O*-Acetylvindoline

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16-*O*-Acetylvindoline (**1a**) was oxidatively transformed into an iminium derivative (**2a**) by copper oxidases (laccase and human ceruloplasmin), an unknown enzyme system(s) of *Streptomyces griseus*, and the chemical oxidizing agent 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The iminium derivative (**2a**) was isolated from enzymatic and chemical oxidation mixtures and was identified by spectral and chemical techniques. Reduction of the iminium compound with sodium borodeuteride provided monodeuterated 16-*O*-acetylvindoline (**1b**) as the sole product. Mass spectral analysis indicated that the deuterium atom was introduced into position C-3 of the piperidine portion of the alkaloid structure. The location and stereochemistry of the deuterium atom were confirmed by high-field ^1H and ^2H NMR analyses of the deuterated product to be in the $^2H_\alpha$ orientation. Hydrolysis of the 16-*O*-acetyl functional group from the iminium derivative (**2a**) resulted in the production of a previously identified dimer (**5**), which forms by intramolecular etherification through the reactive enamine (**3**). The iminium derivative (**2a**) reacts with cyanide to provide complex mixtures of products, one of which was identified by mass spectrometry as a cyanide addition product. The results confirm the existence of a reactive iminium intermediate formed by all of the biochemical and chemical systems examined.

Vindoline (**1**) is one of the major alkaloids of the plant *Catharanthus roseus*. The structure of this toxic compound is found intact in so-called "dimeric" alkaloids, such as vincristine and vinblastine, which have long been used in the treatment of human neoplastic diseases. These complex alkaloids function through their specific ability to achieve metaphase arrest, but little is known of the molecular events associated with specific chemical interactions of these compounds with macromolecules. Indirect evidence has been used to demonstrate that other nitrogen heterocyclic compounds, such as nicotine, benzylpyrrolidine, and phencyclidine,[1-3] yield iminium derivatives

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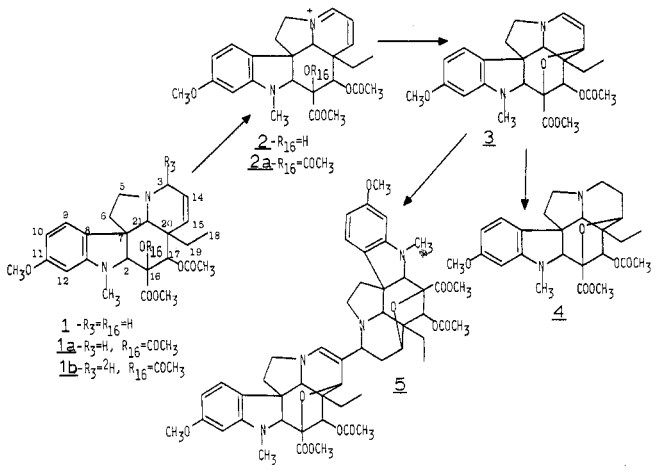
Scheme I. Pathways Operating in the Oxidative Transformation of Vindoline (1) and 16-*O*-Acetylvindoline (1a)



when subjected to metabolic oxidation by microsomal monooxygenases. Reactive electrophilic iminium species generated by metabolic transformation are capable of interacting with biologically important macromolecules, such as nucleic acids, enzymes, and other functional proteins.

Vindoline is the most abundant and available of the *C. roseus* alkaloids and is known to react with copper oxidases, including human ceruloplasmin and plant and fungal laccases,[4] and with undefined enzyme systems of the bacterium *Streptomyces griseus*.[5,6] All of these biochemical systems transform vindoline into the enamine dimer (**5**)[5] and a monomeric enamine precursor to the dimer (**3**),[6] as shown in Scheme I. Although compounds **3** and **4** have been isolated and fully characterized, their presumed precursor iminium (**2**) has never been obtained. Once **2** is formed metabolically, it readily undergoes intramolecular etherification linking positions 15 and 16 of the alkaloid.

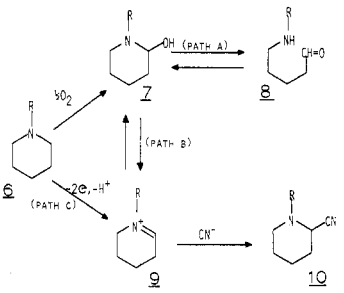
The existence and properties of iminium intermediates are normally demonstrated by indirect methods, such as cyanide trapping, or reduction of the reactive iminium compound with various hydride reagents. In order to obtain firm evidence for the actual existence of an iminium species formed during vindoline biotransformation, 16-*O*-acetylvindoline (**1a**) was selected as a substrate of choice. In **1a**, the protected 16-hydroxyl group of vindoline could not undergo intramolecular etherification, and the resulting iminium intermediate (**2a**) would be expected to accumulate.

We report on the isolation and characterization of **2a**, which is formed by the copper oxidases human ceruloplasmin and *P. anceps* laccase; a chemical mimic of these copper oxidases, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ); and *Streptomyces griseus* cells.

Results and Discussion

Many drugs, industrial chemicals, and naturally occurring compounds are metabolically transformed into chemically reactive intermediates by the action of enzyme systems, such as the monooxygenases of mammalian liver.[7,8] Among these, nitrogen-containing compounds frequently undergo metabolic oxidation at carbons bonded directly to nitrogen to initially form chemically unstable carbinolamines. Carbinolamines are usually not isolated because of their tendency to form aminoaldehydes (Scheme II, path a) or iminium species by loss of hydroxide (Scheme II, path b).[1-4,9] Quaternary iminium electrophiles are important because they may react with nucleophiles like cyanide or with biologically important macromolecules.[1]

Scheme II. Metabolic Pathways Typical in the Oxidation of Nitrogen Heterocyclic Compounds Such as *N*-Methylpiperidine (6)



In our laboratory, copper oxidases, including human serum ceruloplasmin and the laccases,[10,11] and the metabolically prodigious microorganism *Streptomyces griseus* have been used to elaborate the course of oxidation of vindoline[4-6] and 14,15-dihydrovindoline.[9,12] Unlike monooxygenases, the copper oxidases catalyze the oxidation of amines by direct removal of electrons and protons as the first step[4,13,14] (Scheme II, path c). With these enzyme systems, carbinolamines are only formed by the addition of water to iminium species. With compounds like vindoline, position 3 (Scheme I) is most susceptible to the action of copper oxidases, and we have presented evidence to support the involvement of radical-cation intermediates leading to putative iminium derivatives like **2** in the oxidation process. Compounds like **2** are highly unstable and have never been isolated and identified.

16-*O*-Acetylvindoline (**1a**) was selected for study with copper oxidases and *S. griseus* in order to trap and identify **2a** as a product of enzymatic oxidation. If formed, **2a** would likely accumulate because intramolecular etherification to form **3** would be blocked by the 16-*O*-acetyl functional group. As a chemical model for enzyme reactions, 16-*O*-acetylvindoline was oxidized with DDQ to afford **2a** in 56% yield after preparative layer chromatography. The structure of iminium **2a** was supported by IR, NMR, and mass spectral analyses. An N=C stretching band (1660 cm^{-1}) was evident in the IR spectrum. Most proton signals in the NMR spectrum of **2a** were directly comparble to those of vindoline and 16-*O*-acetylvindoline (**1a**). However, olefinic proton signals at 5.18 and 5.84 ppm for H-15 and H-14, respectively, in **1a** were absent in the spectrum of **2a**. These were replaced by broad signals at 8.85 (H-3) and 6.60 ppm (H-14 and H-15 overlapped),

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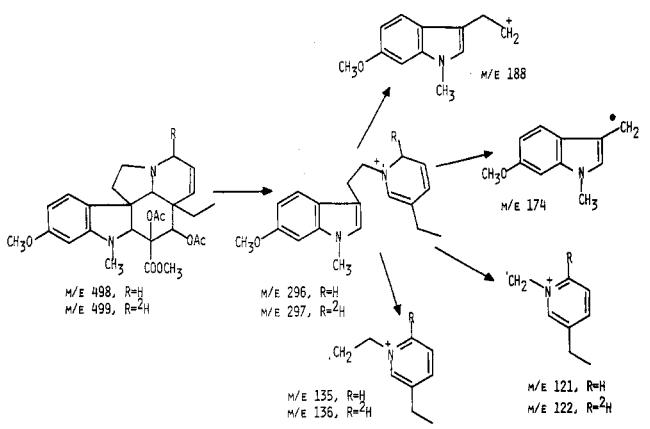
Table I. Analysis of Proton NMR Spectral Data for 16-*O*-Acetylvindoline (**1a**) and 3α-Deuterio-16-*O*-acetylvindoline (**1b**)

	16-*O*-acetylvindoline (**1a**)			3α-deuterio-16-*O*-acetylvindoline (**1b**)		
proton	chem shift, ppm	multiplicity	coupling constant,[a] Hz	chem shift, ppm	multiplicity	coupling constant,[a] Hz
H-14	5.84	ddd	$^3J_{HH}(14,15) = 10.0$ $^3J_{HH}(14,3\beta) = 5.1$ $^3J_{HH}(14,3\alpha) = 2.0$	5.84	dd	$^3J_{HH}(14,15) = 10.0$ $^3J_{HH}(14,3\beta) = 5.1$ $^3J_{HH}(14,3\alpha) = 0$
H-15	5.22	d	$^3J_{HH}(14,15) = 10.0$	5.22	d	$^3J_{HH}(14,15) = 10.0$
H-3β	3.30	dd	$^2J_H(3\beta,3\alpha) = 15.7$ $^3J_{HH}(3\beta,14) = 5.1$	3.30	d	$^3J_{HH}(3\beta,14) = 5.1$
H-3α	2.67	dd	$^2J_{HH}(3\alpha,3\beta) = 15.7$ $^3J_{HH}(3\alpha,14) = 2.0$	*b*		

[a] The superscript 2 or 3 indicates the two-bond or three-bond coupling constant. [b] No signal evident.

Scheme III. Mass Spectral Fragmentation Patterns of 16-*O*-Acetylvindoline (**1a**) and 3α-Deuterio-16-*O*-acetylvindoline (**1b**)



respectively. Imine proton signals similar to that for H-3 in **2a** normally resonate between 8.3 and 9.2 ppm in compounds like 1-methylpyridinium iodide, 3-amino-1-methylpyridinium bromide, pyridine 1-oxide hydrochloride, and several other compounds with the CH=N functional group.[15]

Mass spectral fragmentation patterns provide abundant information about the structures of aspidosperma alkaloids like 16-*O*-acetylvindoline.[16,17] Confirmation of the location of the iminium functional group of **2a** was made by reducing it with $NaBD_4$ in CH_3OD to obtain a product, **1b**, labeled with a single deuterium atom (molecular ion *m/e* 499, 4.8%, Scheme III). Key fragments at *m/e* 136 and 122, which derive from the ion at *m/e* 297, show that the deuterium atom is located in the piperidine ring of 16-*O*-acetylvindoline. Fragments at *m/e* 174 and 188 were not accompanied by the intense M + 1 ions expected if deuterium was located in either the indole ring or the ethylene side chain found in these fragments.

The precise location of deuterium at position 3 follows from chromatographic and spectral similarities of **1b** to **1a**. With $NaBD_4$, deuteride could attack either position 3 or 15. However, only deuteride attack at position 3 could provide **1b** as product. Deuteration at position 15 would result in formation of an unstable enamine like **3** (Figure 1), which we previously indentified[6] as a precursor to enamine dimers like **5**. High-field ^1H and ^2H NMR analyses (Figure 1a,b) confirmed the location and stereochemistry

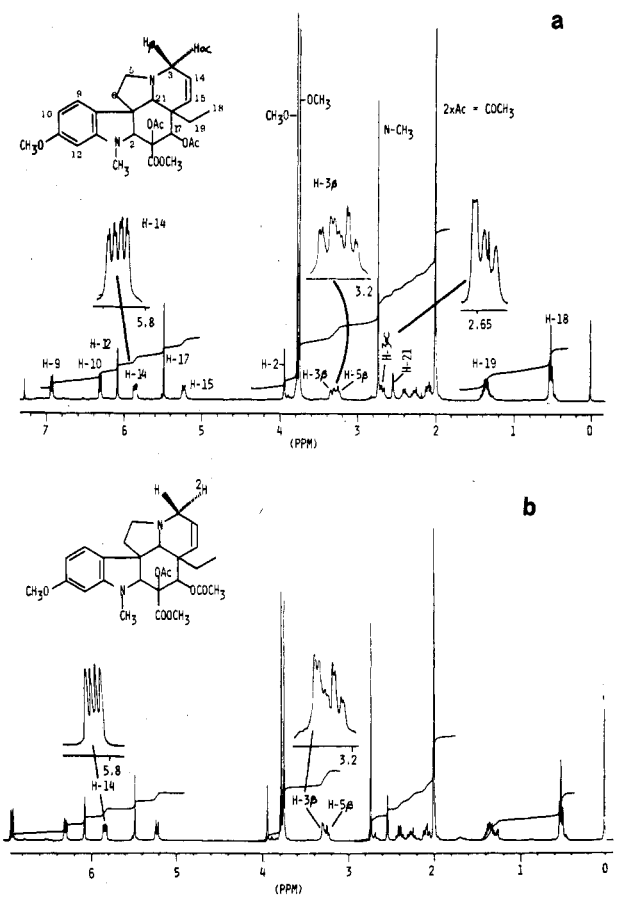


Figure 1. The 360-MHz ^1H NMR spectrum of (a) 16-*O*-acetylvindoline (**1a**) and (b) 3α-deuterio-16-*O*-acetylvindoline (**1b**).

of the deuterium atom at position 3. The ^1H NMR spectral properties of vindoline at 360 MHz have been reported,[18] and we have observed identical spectra (with a similar instrument) for this compound and 16-*O*-acetylvindoline. Proton signal multiplicities and coupling constants give a clear picture of the relative positions of each proton attached to carbons 3, 14, and 15 of **1a** and 3α-deuterio-16-*O*-acetylvindoline (**1b**), and these have been confirmed by decoupling experiments (Table I). Measured dihedral angles for protons at 3β/14 (44°) and 3α/14 (77°) for the preferred conformer of 16-*O*-acetylvindoline are consistent with observed coupling constants of 5.1 and 2.0 Hz, respectively, for these vicinal protons.

In the spectrum of **1b**, geminal coupling between H-3β and H-3α was absent, and the signal for H-3β occurred as a doublet at 3.30 ppm with $J = 5.1$ Hz due to coupling only with H-14. The signal for H-3α was absent in the spec-

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Table II. Mass Spectral Analysis of **1b** Produced by $NaBD_4$ Reduction of **2a** Formed by DDQ, Copper Oxidases (Ceruloplasmin and Laccase), and *Streptomyces griseus* Oxidations of 16-*O*-Acetylvindoline (**1a**)

	percent relative abundances				
m/e	16-*O*-acetylvindoline (**1a**)	DDQ (**1b**)	ceruloplasmin (**1b**)	laccase (**1b**)	*S. griseus* (**1b**)
499	0.7	4.8	1	3.7	2.5
498	1.6	0.4		0.2	0.3
297	1.4	12.4	14.1	8.2	11.8
296	4.3	5.4	4.5	3.4	6.2
189	33.9	41.2	44.9	40.8	42.2
188	38.4	47.4	57.8	42.9	68.7
175	4.2	9	13.5	10.8	16.7
174	26.1	33.2	38.2	26.8	50
136	11.3	100	100	100	100
135	100	78	64.8	67.2	65
122	21.6	51.2	50.5	56.9	52.9
121	51.4	30.2	31.8	31	32.9

trum, and the signal at 5.84 ppm for proton H-14 was a doublet of doublets due to couplings only between protons H-3β and H-15. These results clearly fix the position of the deuterium atom at 3α, the expected position if $NaBD_4$ reduction occurred from the least hindered face of iminium **2a**. Deuterium NMR spectroscopy gave a single signal at 2.58 ppm, which compares well with the proton chemical shift of 2.67 ppm for the H-3α proton in the ^1H NMR spectrum. The facile and complete reduction of the iminium **2a** to **1b** provided us with a powerful and specific probe to determine whether iminium compounds are formed during the oxidation of 16-*O*-acetylvindoline by copper oxidases and *S. griseus*.

Preparative scale incubations of 16-*O*-acetylvindoline (**1a**) with human serum ceruloplasmin, *Polyporus anceps* laccase, and *S. griseus* resting cells provided yields (TLC estimates) of 100% in 1 h, 50% in 5 h, and 20% in 24 h, respectively. No other products were formed in any of the incubations. Since reactions were incomplete with ceruloplasmin and *S. griseus*, preparative layer chromatography was used to remove unreacted starting material before $NaBD_4$ reductions were accomplished. Reduction of enzyme and cell-incubation polar products was accomplished with $NaBD_4$ in CH_3OD to provide 3α-deuterio-16-*O*-acetylvindoline (**1b**) (TLC, HPLC) in each case, and the deuterated products were subjected to mass spectral analyses (Table II, Scheme III). All products contained a single deuterium atom in the piperidine ring as evidenced by M + 1 peaks at *m/e* 136 and 122 and by the lack of an M + 1 peak associated with fragments at *m/e* 174 and 188. These results indicate that the polar product formed by enzyme and cell systems was the iminium derivative **2a**.

Attempts to trap **2a** formed in enzyme reactions by cyanide were unsuccessful. Only one of several products formed with cyanide provided mass spectral evidence (*m/e* 523 expected), suggestive of a cyanide addition product. Due to limited amounts of product available and complexities of reaction mixtures, further spectral analysis was not possible. Thus, the specific site of cyanide addition remains ambiguous.

The final verification of **2a** as a trapped iminium intermediate in the pathway of vindoline oxidation by laccase was obtained by cleavage of the 16-*O*-acetyl functional group with sodium methoxide. The resulting highly unstable iminium derivative **2** undergoes spontaneous intramolecular etherification to enamine **3** (TLC) and the dimer **5** (TLC, HPLC), both of which are formed when vindoline is incubated with copper oxidases[4] or *S. griseus*.[5,6]

These findings are significant in providing direct evidence for the existence of iminium intermediates in the metabolism of nitrogen heterocycles like vindoline. The iminium **2a** is remarkably stable in aqueous media and can be isolated by preparative layer chromatography. The facile reduction of **2a** with hydride reducing agents underlines the potential for such compounds to react with other nucleophiles that, perhaps, are of biological significance. While the oxidation pathway of the copper oxidases is well-established, less is known of the enzyme systems of *S. griseus*, which catalyze transformations of nitrogen heterocycles,[5,6] and active cell-free preparations have not been successfully isolated from this organism.[19] We hope to extend our findings with vindoline to other clinically significant nitrogen heterocycles, which are also metabolically transformed by copper oxidases.

Experimental Section

Infrared spectra were determined on a Perkin-Elmer 267 grating IR spectrophotometer, and UV spectra were determined on a Phillips Pye Unicam SP1800 spectrophotometer. Proton nuclear magnetic resonance (^1H NMR) spectra were obtained at 60 MHz with a Varian EM-360-A at 360 MHz with a Bruker WM-360 FTQNMR, or at 500 MHz with a Bruker WM500 FTNMR spectrometer with $CDCl_3$ or CD_3OD as solvents and with tetramethylsilane as an internal standard. Data processing for low-power decoupling experiments (setting of 10-L) were accomplished with the Bruker Aspect 2000 computer and an Aspect 2000 pulse programmer. Deuterium magnetic resonance spectra were determined on a JEOL FX-90-Q instrument equipped with a Nicolet data system using an external ^7Li, 34.84-MHz lock. The spectrometer frequency for observation of deuterium nuclei was 13.755 MHz using a sweep width of 200 Hz. There were 240 pulses acquired at a pulse width of 1.28 s for deuterated 16-*O*-acetylvindoline, which was dissolved in $CHCl_3$ (spectral grade) at a concentration of 0.120 mM. The reference was provided by 0.12 mM $CDCl_3$ in chloroform. Low-resolution mass spectra were obtained on a Finnigan Model 3200 spectrometer or a Ribermag R-10-10C, Nermag-S-a, France, spectrometer. Chemical-ionization mass spectra were obtained on the latter instrument with ammonia as the reagent gas.

Chromatography. Thin-layer chromatography (TLC) was performed on 0.25- or 0.5-mm layers of silica gel GF_{254} (Merck) prepared on glass plates. Plates were air-dried and oven activated at 120 °C for 30 min prior to use. The following solvent systems were used: A, ethyl acetate/methanol (3:1) (vindoline, R_f 0.7; 16-*O*-acetylvindoline, R_f 0.82; dimer, R_f 0.8); B, ethyl acetate (100%) (vindoline, R_f 0.4; 16-*O*-acetylvindoline, R_f 0.8; dimer, R_f 0.65); C, chloroform/ethanol (30:1) (16-*O*-acetylvindoline, R_f 0.6; cyanide adduct **1c**, R_f 0.7). Compounds were detected on developed chromatograms by fluorescence quenching under 254-nm UV light and were later visualized by spraying with cerium(IV) ammonium sulfate (1% in 50%, v/v, H_3PO_4).[20] High-performance liquid chromatography (HPLC) was performed as described elsewhere,[4] and separations were best achieved with a μBondapak phenyl column (0.4 × 30 cm, waters) with acetonitrile/aqueous 0.005 M $(NH_4)_2HPO_4$ (65:35) as solvent at a flow rate of 1 mL/min at an operating pressure of 1400 psi. Authentic standards were singly injected in order to establish individual retention volumes, and mixtures of all metabolites were well-resolved. The identities of individual metabolite peaks were confirmed by "spiking" with analytical standards. Retention volumes of various compounds were as follows: vindoline (**1**) 4.80 mL; 16-*O*-acetylvindoline (**1a**), 5.76 mL; enamine dimer (**5**), 7.30 mL.

Materials. Vindoline (**1**) was provided by the Eli Lilly Co., Indianapolis, IN, and the enamine dimer **5** was an authentic standard available from previous work in our laboratory.[5,6] The purities and identities of all compounds were established by chromatography, high- and low-resolution mass spectrometry, and

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^{13}C and ^{1}H NMR spectroscopy before use.

16-*O*-Acetylvindoline (1a). 16-*O*-Acetylvindoline (**1a**) was prepared by dissolving 4.6 g of vindoline in 200 mL of pyridine and 250 mL of acetic anhydride. The reaction was stirred under a nitrogen atmosphere at 40 °C for 48 h and was stopped by pouring over 1 kg of ice. The resulting solution was adjusted to pH 9.5 with 58% NH_4OH and filtered to provide 3.3 g (72% yield) of crude **1a**. Recrystallization from methanol gave 16-*O*-acetylvindoline (**1a**), which displayed the following properties: mp 134–135 °C; UV λ_{max} (EtOH) 217 nm (log ϵ 4.60), 252 (3.86), 304 (3.71); for ^{1}H NMR (360 MHz, $CDCl_3$) data, see Figure 1a; for mass spectral data, see Table I. Anal. Calcd for $C_{27}H_{34}N_2O_7$: C, 65.04; H, 6.87; N, 5.62. Found: C, 64.82; H, 7.06; N, 5.79.

Oxidation of 16-*O*-Acetylvindoline (1a) to Compound 2a with 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). DDQ (600 mg) was added to a solution of 1 g of **1a** in 500 mL of methanol, and the mixture was stirred at room temperature for 15 min. At this time, most of the starting material was converted to a single polar product at R_f 0.1 (solvent system A). The mixture was concentrated to dryness under reduced pressure, and the resulting brown solid was dissolved in a small volume of methanol and immediately applied to 0.5-mm silica gel GF_{254} preparative layer plates and developed in solvent system A. The band centered at R_f 0.1 containing the polar product was scraped from the plates and eluted from silica gel by several rapid washes in methanol. The collected eluates were concentrated and dried under vacuum to provide 567 mg of the polar product, which possessed the following physical properties: chemical-ionization, mass spectrum, *m/e* (relative abundance) 498 (42.7), 497 (100), 296 (5.3), 295 (16), 188 (89.4), 174 (6.6), 136 (23.6), 135 (5.4), 122 (9.4), 121 (5.2); UV λ_{max} (MeOH) 251 nm (log ϵ 4.07), 301 (3.77); ^{1}H NMR (500 MHz, CD_3OD) 0.65 (t, 3 H, CH_3CH_2), 1.28 (q, 2 H, CH_3CH_2), 1.93 (br s, 6 H, 2 $CH_3C{=}O$), 2.75 (s, 3 H, NCH_3), 3.62 (s, 3 H, OCH_3 or $COOCH_3$), 3.72 (s, 3 H, OCH_3 or $COOCH_3$), 3.85 (s, 1 H, H-2), 5.2 (s, 1 H, H-17), 6.24 (d, J = 2 Hz, 1 H, H-12), 6.31 (dd, J = 8 and 2 Hz, 1 H, H-10), 6.60 (br s, 2 H, H-14 and H-15), 7.12 (d, J = 8 Hz, 1 H, H-9), 8.85 (br s, 1 H, H-3) ppm.

Reduction of the Iminium Derivative 2a with $NaBD_4$ in CH_3OD. The polar iminium derivative **2a** (200 mg) was dissolved in 5 mL of CH_3OD and reduced by the addition of 100 mg of $NaBD_4$. After 30 min, TLC (solvent system A) indicated that the reaction was complete and that the polar compound had been quantitatively converted to a single product possessing R_f 0.82 by TLC (solvent system A) identical with 16-O_5acetylvindoline. The reaction mixture was evaporated to dryness under a stream of nitrogen, the resulting solid residue was dissolved in 20 mL of water, and the mixture was extracted three times with 20 mL of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous $MgSO_4$ before being concentrated under a stream of nitrogen. After thorough removal of solvent under high vacuum, 152 mg of a solid was obtained. This solid (111 mg) was further purified by column chromatography using a 1.5 × 25 cm column slurry-packed with silica gel (Baker 3204, 60-200 mesh) in the developing solvent of ethyl acetate/benzene (1:1). The column was eluted at a flow rate of 0.3 mL/min while fractions of 0.7 mL were collected. Those containing the pure deuterated 16-*O*-acetylvindoline derivative (**1b**, Scheme I) (fractions 14–45) were combined and concentrated to dryness. The analytically pure sample of **1b** (30 mg) was used for ^{1}H and ^{2}H NMR and mass spectral analyses. This sample of **1b** was chromatographically [TLC (solvent system A) and HPLC] identical with 16-*O*-acetylvindoline. The mass spectrum was identical with **1a**, with appropriate fragments containing a single atom of deuterium, as shown in Table II and Scheme III. The ^{1}H NMR spectrum of **1b** at 360 MHz was taken in $CDCl_3$ and is shown in Figure 1b. The ^{2}H NMR spectrum of **1b** was obtained in $CHCl_3$ to display a single absorption peak for deuterium centered at 2.58 ppm.

Enzymes. *Polyporus anceps* laccase was produced and isolated in our laboratory as previously described,[21] and enzyme activities were standardized by a spectrophotometric assay procedure using syringaldazine as substrate.[4,21] Laccase incubations were prepared by adding 2 mg (4.02×10^{-6} mol) of 16-*O*-acetylvindoline (**1a**) in 0.1 mL of MeOH to 10 mL of pH 5.0, 0.2 M potassium phosphate solution containing 1.04 units of laccase and 9.4 mg (2.65×10^{-6} mol) of chlorpromazine as a cofactor. Under these conditions, reactions are instantaneous as determined with a Clark oxygen electrode.[4]

Human ceruloplasmin (Sigma Chemical Co., type III) purity was determined by measuring the ratio of absorbances at 610/280 nm as 0.046. Pure crystalline ceruloplasmin has a measured ratio of 0.047.[4,13] Enzyme concentrations were determined from the 610-nm absorption peak (ϵ 10900 M)[22] based on a molecular weight of 132000.[23] Ceruloplasmin activity was standardized in international units by the method of Curzon et al.[24] Ceruloplasmin oxidation reactions were conducted by adding 2 mg of 16-*O*-acetylvindoline (**1a**) in 0.1 mL of MeOH to 5.075 mL of pH 5.5, 0.2 M acetate buffer containing 562.5 units of ceruloplasmin and 9.4 mg of chlorpromazine.

Analytical scale enzyme incubations were conducted in 50-mL Delong flasks shaken at 250 rpm and 27 °C in a New Brunswick Scientific Co. Model G24 gyrotory incubator shaker. Control incubations consisted of buffers with enzyme alone, substrate alone, substrates plus cofactors, and complete incubations containing boiled enzymes. Reactions were observed only in complete enzyme incubation mixtures.

Enzyme reactions were routinely monitored by withdrawing 1- or 2-mL samples at various time intervals, adjusting these to pH 9.5 with NH_4OH, and extracting with half volumes of ethyl acetate. Extracts were examined by TLC using solvent system A and by HPLC.

***Streptomyces griseus* Incubations.** Methods used in the cultivation of *S. griseus* (UI 1158, NRRL B8090) and in the use of resting cell preparations with this organism have been described elsewhere.[12,25] In general, the organism is cultivated in a two-stage incubation procedure in a soybean meal–glucose medium.[4,12,25] After the organisms are allowed to grow for 24 h, second stage cultures are harvested by filtration of cell suspensions through cheese-cloth to remove unwanted solids, followed by centrifugation at 8000*g* for 10 min (Sorvall RC5 refrigerated centrifuge). The resulting *S. griseus* cell paste is stored in glass containers at −60 °C until required for use.

Resting-cell incubations are conducted by suspending thawed *S. griseus* cells in 0.1 M sodium phosphate buffer, pH 6.5. Buffer volumes were adjusted to allow for three times the numbers of *S. griseus* cells normally found in fermentation media. The substrate, 16-*O*-acetylvindoline (**1a**) (10 mg dissolved in 0.1 mL of dimethylformamide), was added to 25-mL volumes of cell suspensions held in 125-mL Delong culture flasks. Substrate-containing cultures were shaken at 250 rpm at 27 °C, and 4-mL samples were taken at various time intervals. Samples were adjusted to pH 9.5 with 58% NH_4OH and extracted with 1 mL of ethyl acetate, and the extracts were examined by HPLC or TLC (solvent system A) analyses.

Formation and Trapping of an Iminium (2a) Derivative of 16-*O*-Acetylvindoline by Ceruloplasmin, Laccase, and *S. griseus* Cells. (a) *P. anceps* Laccase. 16-*O*-Acetylvindoline (**1a**, 32 mg) and chlorpromazine (53 mg) were added to 160 mL of pH 5.0, 0.2 M potassium phosphate solution containing 20 units of laccase. The reaction mixture was divided among six 125-mL Delong flasks and incubated with shaking. TLC monitoring of the reaction indicated that a polar (R_f 0.1, solvent system A) compound with chromatographic properties identical with **2a** prepared by DDQ oxidation of 16-*O*-acetylvindoline was formed immediately and that the reaction was complete within 1 h. The combined reaction mixture was adjusted to pH 9.5 with NH_4OH and extracted three times with 80 mL of ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. This residue was dissolved in 13 mL of CH_3OD and reduced with $NaBD_4$ as described earlier. The resulting deuterated 16-*O*-acetylvindoline (**1b**) was subjected to preparative TLC (0.5 mm layer) using solvent system A to provide 5.5 mg of the analytical sample. This product was subjected to mass

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spectral analysis, and the results are shown in Table II.

(b) Ceruloplasmin. A preparative-scale incubation was conducted by adding 120 mg of 16-*O*-acetylvindoline (**1a**) and 180 mg of chlorpromazine to 600 mL of pH 5.5, 0.2 M sodium acetate buffer containing 3240 units of ceruloplasmin. This mixture was incubated at 37 °C, and the polar (R_f 0.1, solvent system A) metabolite was formed immediately. At 5 h, the transformation reaction was estimated to be 50% complete (TLC), and it was terminated by adjusting the pH to 9.5 with NH_4OH and extracting with ethyl acetate as before. The extract was subjected to preparative TLC with 0.5-mm layer silica gel GF_{254} plates and solvent system A to obtain the polar metabolite free from contaminating 16-*O*-acetylvindoline. The presumed iminium derivative (**2a**) was dissolved in 20 mL of CH_3OD and reduced with $NaBD_4$ as described earlier, and the resulting analytically pure product (2 mg) was subjected to mass spectral analysis (Table II).

(c) *S. griseus* Cells. 16-*O*-Acetylvindoline (**1a**) was also incubated with *S. griseus* cell suspensions. A total of 130 mg of **1a** in 10 mL of MeOH was added to 400 mL of *S. griseus* cell suspension, and the mixture was incubated for 24 h. At this stage, approximately 20% of the substrate **1a** was converted to a single polar product, and the reaction was terminated by adjustment of the pH to 9.5 with NH_4OH and extraction with ethyl acetate. Preparative layer chromatography was used to remove unreacted **1a** from the presumed polar iminium metabolite **2a**. The polar metabolite was then dissolved in CH_3OD and reduced with $NaBD_4$ as described before, and the resulting deuteriated 16-*O*-acetylvindoline (0.6 mg) (**1b**) was subjected to mass spectral analysis (Table II).

Conversion of an Acetyl Iminium Derivative (2a) to the Enamine Dimer (5). 16-*O*-Acetylvindoline (**1a**; 10 mg in 0.1 mL MeOH) was added to each of three flasks containing 25 mL of pH 5.0, 0.2 M potassium phosphate solution and 15 units of *P. anceps* laccase. The contents of all the flasks were pooled, adjusted to pH 9.5, and extracted with an equal volume of ethyl acetate. The extracts were dried under vacuum, redissolved in 2 mL of 0.5 N sodium methoxide, and allowed to react on ice with stirring under a stream of nitrogen. The reaction was quenched after 7 h by the addition of 10 mg of Na_2HPO_4 and 5 mL of water. The resulting solution was extracted with chloroform (2 × 10 mL), dried over anhydrous Na_2SO_4, and evaporated under vacuum to a pale yellow residue. The residue was further purified over 0.5-mm silica gel GF_{254} developed with solvent system B. The band between R_f 0.60 and 0.85 was scraped from the plate and eluted with acetone to provide a pale yellow residue upon evaporation. The residue contained a mixture of vindoline (**1**) and the enamine dimer (**5**) by TLC (solvent systems A and B) and by HPLC when compared with authentic standards.

Trapping of the Iminium Derivative (2a) with Cyanide Ion. 16-*O*-Acetylvindoline (**1a**; 10 mg in 0.1 mL MeOH) was added to each of five flasks containing 25 mL of pH 5.0, 0.2 M potassium phosphate solution and laccase (23 units), and incubations were conducted as before. The reactions were terminated by adjustment to pH 9.5 with NH_4OH and extraction with 100 mL of ethyl acetate. The extract was evaporated to dryness and redissolved in 3 mL of saturated, methanolic KCN. The cyanide-containing mixture was stirred for 4 h at room temperature before being poured into 3 mL of a saturated solution of NaCl, which was then extracted with chloroform (3 × 10 mL). The organic extracts were pooled and dried to yield 55 mg of crude red oil. TLC of the oil (solvent system C) revealed at least four products plus unreacted starting material. Controls of 16-*O*-acetylvindoline stirred in saturated methanolic KCN showed no product formation by TLC. The oil was purified by passing it through a minicolumn of silica gel (7 g, 1 × 20 cm) using chloroform as eluant, while 3 mL fractions were collected. Fractions 1 and 2 were identical and were combined to yield a product of lower polarity than 16-*O*-acetylvindoline (system C) (2 mg), which was subjected to mass spectral analysis: *m/e* (relative intensity) 523 (1), 495 (1), 496 (1), 467 (1), 453 (1), 437 (1), 377 (2), 365 (4), 320 (3), 295 (7), 293 (5), 214 (6), 213 (10), 212 (14), 188 (100), 174 (78), 135 (22), 121 (24).

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Acetylation of Some Novel Hemicholinium Compounds by Soluble Choline Acetyltransferase: Structure–Activity Relationships

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Four bisquaternary nitrogen analogues of 2,2′-[1,1′-biphenyl]-4,4′-diylbis[2-hydroxy-4,4-dimethylmorpholinium] bromide (hemicholinium 3, HC-3) have been synthesized. These analogues differ from HC-3 in that they have a number of methylene groups inserted between the two phenyl rings. This study examines the significance of the internitrogen distance in these compounds with regard to their acetylation by soluble choline acetyltransferase (ChAc) in vitro. The hemicholinium compounds were incubated with [^{14}C]acetylcoenzyme A and any acetylated products were isolated by liquid ion exchange. Only HC-3 and the analogue with three methylene groups between the two phenyl rings, that is, 2,2′-(1,3-propanediyldi-1,4-phenylene)bis[2-hydroxy-4,4-dimethylmorpholinium] (3CHC), were found to be significantly acetylated. The acetylation rate of both these two compounds was 28% that of choline. It is concluded that an internitrogen distance of 14 Å in bisquaternary nitrogen choline analogues provides the optimum distance for acetylation by ChAc in vitro.

Choline is an essential substrate for the synthesis of acetylcholine in cholinergic neurons. This synthesis is catalyzed by the enzyme acetylcoenzyme A:choline *O*-acetyltransferase (EC 2.3.1.6, choline acetyltransferase, ChAc), the properties of which have recently been reviewed and discussed.[1-5] A partially purified preparation of this

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