MICROBIOLOGICAL O-DEMETHYLATION OF DIHYDROVINDOLINE

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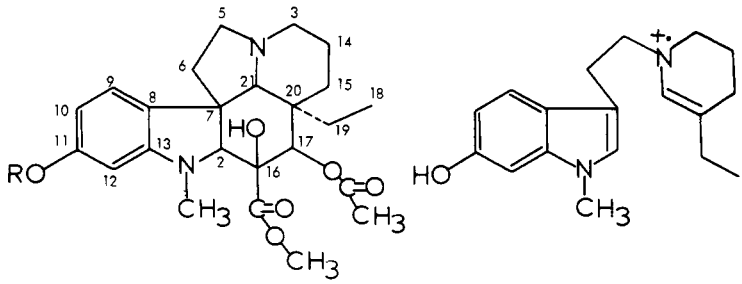
Microbiological transformation studies have been conducted with vindoline and several of its derivatives in our laboratories. We were interested in learning more about pathways by which *Streptomyces griseus* (UI 1158, NRRL B 8090) achieved oxidations of vindoline to metabolites like dihydrovindoline ether, and a dihydrovindoline ether dimer (1, 2). To this end, dihydrovindoline (**1a**) was prepared as a substrate and incubated with growing cultures and resting cell suspensions of *S. griseus* to provide complex mixtures of metabolites as observed by thin layer chromatographic (tlc) and high-performance liquid chromatographic (hplc) analyses. A preparative scale incubation employing 1.95 liters of resting cells of *S. griseus* in 0.2M phosphate buffer, pH 6.1, and 780 mg of (**1a**) as substrate gave the major metabolite in approximately 10% yield after extensive column chromatographic purification. The structure of the dihydrovindoline metabolite was based on an evaluation of its spectral properties.

A broad –OH stretching band between 3600 cm^{-1} and 3100 cm^{-1} in the infrared spectrum and a bathochromic shift in the absorption peak at 306 nm in the uv spectrum in the presence of 1% NaOH both suggested a phenolic nature for the metabolite. No corresponding bathochromic shift is observed when dihydrovindoline itself is treated with base. The metabolite was also insoluble in $NaHCO_3$ but was readily soluble in 5% NaOH, suggesting that the metabolite was not a carboxylic acid derivative.

The ^1H nmr spectrum of the metabolite was nearly identical to that of dihydrovindoline except for the absence of a singlet at 3.78 ppm which is attributable to either the three methyl group protons of the methyl ether or of the methyl ester. The *N*-methyl group signal was clearly evident at 2.60 ppm in the structure of the metabolite, thus ruling out the possibility that the metabolite might be an *N*-demethylated derivative. ^{13}C nmr spectra for the metabolite and for dihydrovindoline (3) were also nearly identical with the exception of a single signal. The signal at 55.0 ppm for the aromatic methoxyl carbon atom is absent in the spectrum of the metabolite.

The metabolite behaved much like vindoline (4), dihydrovindoline (4) and *O*-desmethylvindoline (5) in the mass spectrometer, and it possessed a molecular ion of *m/e* 444 for $C_{24}H_{32}N_2O_6$ in the high resolution mass spectrum. The molecular weight of the metabolite indicated a loss of a methyl group vs. dihydrovindoline. A major fragmentation of the metabolite occurs by well-known pathways for these alkaloids and results in an effective separation of ions containing the methyl ether or methyl ester portions of the metabolite (figure 1). The peak at *m/e* 284 (**2**) consists of the indole ring system minus carbons 16 and 17 plus their substituents (4, 5). This fragment clearly indicates that the methyl group had been metabolically cleaved from the indole portion of the molecule.

All of the spectral and physical evidence strongly support the structure of the metabolite as 11-*O*-desmethyldihydrovindoline (**1b**). Vindoline, which contains a 14,15-double bond, is metabolized extensively by *Streptomyces griseus*, but only to products obtained by oxidation reactions centered in the six-membered heterocyclic ring (1, 2). We previously reported the conversion of vindoline to 11-*O*-desmethylvindoline with *Sepedonium chryosospermum* (5). However,



1a R CH_3 2

1b R H

FIG. 1. The structures of dihydrovindoline (**1a**) and 11-*O*-desmethyldihydrovindoline (**1b**), and the major mass spectral fragment (**2**) which is obtained from **1b**.

S. griseus is incapable of achieving the *O*-demethylation reaction with vindoline as substrate. We believe that our results with dihydrovindoline represent a good example of the subtle control exerted by a simple functional group like a double bond over the course of biotransformation of a structurally complex substrate.

EXPERIMENTAL

GENERAL METHODS.—Melting points were determined in open-ended capillaries with a Thomas-Hoover apparatus and are uncorrected. Ir spectra were obtained on a Perkin Elmer 267 spectrophotometer with KBr disks and uv spectra were recorded in a Beckman Ratio Recording spectrophotometer. ^{1}H nmr spectra were recorded with a Varian T-60 spectrometer with tetramethylsilane as internal standard. ^{13}C nmr spectra were obtained on a Bruker HX-90E spectrometer at 22.63 MHz incorporating a time-shared internal deuterium lock, a Bruker SXP high power radiofrequency amplifier, a Nicolet BNC-12 computer, and model 293 I/O controller for signal averaging and Fourier transformation of the free induction decay. Spectra were obtained on samples dissolved in deuteriochloroform (0.14M) in 10 mm tubes. High-resolution mass spectral data were provided by the Midwest Center for Mass Spectrometry, University of Nebraska, Lincoln, Nebraska. Vindoline was obtained as a generous gift from Eli Lilly and Co., Indianapolis, Indiana, and has been fully characterized (1).

CHROMATOGRAPHY.—Tlc was performed on 0.25 mm thick layers of silica gel GF_{254} (Merck) on glass plates. Prior to use, plates were activated at 120° for 30 min. Tlc systems used included: $CHCl_3$-MeOH (30:1) -dihydrovindoline (Rf 0.70) -*O*-desmethyldihydrovindoline (Rf 0.50); and $CHCl_3$-MeOH (9:1) -dihydrovindoline (Rf 0.80) -*O*-desmethyldihydrovindoline (Rf 0.60). Visualization of developed tlc plates was accomplished by spraying with cerium (IV) ammonium sulfate (1% in 50% H_3PO_4) (6).

Hplc was performed on a Waters Associates ALC/GPC 202 instrument equipped with an M6000 solvent delivery system, a U6K universal injector, and a 254 nm differential uv detector. Separations were best achieved with a Microbondapak phenyl column (Altech 30 x 4.1 cm) using methanol-0.005M $(NH_4)_2HPO_4$ (6:4) at an average flow rate of 1.0 ml/min at a pressure of 1000 lb/in^2. Retention volumes were: dihydrovindoline, 24.6 ml; O-desmethyldihydrovindoline 11.4 ml. The identities of individual peaks in microbial extracts were confirmed by "spiking" with individual standards.

DIHYDROVINDOLINE (1a).—Vindoline (5.0 g) and 2 g of PtO_2 in 200 ml of absolute ethanol were catalytically hydrogenated on a Parr shaker at 40 psi for six hours. The reaction mixture was filtered and the filtrate evaporated under reduced pressure to yield a white glass (4.98 g, 99%). The glass was recrystallized in methanol giving fine white crystalls of 14,15-dihydrovindoline (**1a**) (7): mp 115–117°C; uv (ETOH) λmax 252 nm (log ε 3.79) and 304 (3.66); ir (KBR) 3600 to 3200, 2950, 2865, 1735, 1610, 1240, 1220, 1035; ^{1}H nmr ($CDCl_3$) ppm, 0.48 (3H, t, J=7 Hz, H-18), 1.35 (2H, m, H-19), 2.1 (3H, s, $COCH_3$) 2.6 (3H, s, N-CH_3), 3.75 (1H, s, H-2), 3.78 (3H, s, OCH_3 or CO_2CH_3), 3.80 (3H, s, OCH_3 or CO_2CH_3), 5.65 (1H, s, H-17), 6.05 (1H, d, J=2 Hz, H-12), 6.03 (1H, dxd, J=8 and 2 Hz, H-10), 6.90 (1H, d, J=8 Hz, H-9) these assignments are largely analogous to those for vindoline (7). Mass spectrum, *m/e* (rel intensity) 458 (<1), 399 (<1), 298 (17), 188 (14), 174 (5), 124 (100). Anal. calcd. for $C_{25}H_{32}N_2O_6$: C, 65.48; H, 7.47; N, 6.11. Found: C, 65.23; H, 7.46; N, 5.96.

FERMENTATION PROCEDURE.—*Streptomyces griseus* (UI 1158, NRRL B 8090) was maintained on ATCC medium #5. Methods used in the cultivation of microorganisms have been described elsewhere (8). A two-stage incubation procedure using a soybean meal-maltose medium was employed. Incu-

bations were conducted on rotary shakers (model G-25 New Brunswick Scientific Co.) operating at 250 rpm and 27°. The substrate, dihydrovindoline, was added to the 24-hour-old stage II culture as a solution in dimethylformamide (DMF) to a final concentration of 400 μg/ml of culture medium. Controls consisted of cultures grown without substrate and of incubations containing medium and substrate without microorganisms. Samples were withdrawn after 24, 48, 72, and 96 h, adjusted to pH 9.0 with 58% NH_4OH, and extracted with ethyl acetate for tlc and hplc analysis. Maximum conversions were seen at 96 h (tlc analysis) for both resting and growing cell incubations.

RESTING CELL INCUBATIONS.—*S. griseus* cultures were grown according to the usual two-stage fermentation scheme using a peptone-beef extract medium. The mycelium of the 24 h second-stage cultures was harvested by centrifugation at 10,444 x g for 10 min (Sorvall RC-5 refrigerated centrifuge) and transferred to 0.2 M K_2HPO_4:KH_2PO_4 buffer at pH 6.1. Incubations were conducted in 125 ml flasks and cell concentrations were made to correspond to three times the normal cell concentrations in 25 ml of 0.2 M phosphate buffer at pH 6.1. Dihydrovindoline was added as a solution in DMF to a final concentration of 400 μg/ml of cell solution. Flasks were incubated and sampled as described above. Controls consisted of cells incubated without substrate and flasks of buffer and substrate without cells.

PREPARATION AND CHARACTERIZATION OF 11-*O*-DESMETHYLDIHYDROVINDOLINE (**1b**).—Resting cells were prepared as described. A total of 780 mg of dihydrovindoline (**1a**) in 7.8 ml of DMF was distributed evenly among 78, 125 ml flasks, each containing 25 ml of resting cell suspension. After 96 h, the incubation mixtures were pooled, adjusted to pH 9.0 with 58% NH_4OH, and extracted with 3 x 500 ml ethyl acetate. The extracts were dried over anhydrous sodium sulfate and concentrated to a viscous brown oil (880 mg). The oil was dissolved in a minimum amount of ethyl acetate, applied to 5 preparative plates of silica gel GF_{254} (Merck), 0.5 mm thick, and the plates were developed with chloroform-ethanol (30:1). The bands at Rf 0.25–0.50 were removed and extracted with ethyl acetate. The extracts, when dried over anhydrous sodium sulfate and evaporated, yielded a yellow-brown oil (110 mg). The oil was dissolved in 1 ml of $CHCl_3$ and applied to a column (11 g Neutral Alumina, 22 x 1 cm) which was eluted with chloroform-ethanol (60:1) at a flow rate of 2 ml/min while 5 ml fractions were collected. *O*-desmethyldihydrovindoline (**1b**) (71 mg) was eluted in fractions 16–18. The analytical sample was prepared by dissolving the compound in 1 ml of chloroform-ethanol (60:1) and applying it to a column (9 g silica gel, 22 x 1 cm) which was eluted with the same solvent system at a flow rate of 0.4 ml/min while 3 ml fractions were collected. Fractions 17–36, when combined and dried in vacuum, yielded *O*-desmethyldihydrovindoline (**1b**) (33.3 mg): uv (ETOH) λmax 252 nm (log ε 3.70), 306 (3.56); ir (KBR) cm^{-1} 3350, 2940, 2860, 1740, 1610, 1500, 1370, 1340, 1240, 1040; ^1H nmr ($CDCl_3$) ppm, 0.48 (3H, t, H-18), 2.02 (3H, s, $COCH_3$), 2.60 (3H, s, NCH_3), 3.7 (1H, s, H-2), 3.8 (3H, s, $OCOCH_3$), 5.6 (1H, s, H-17), 6.0 (1H, d, J=2 Hz, H-12), 6.1 (1H, d x d, J=8 and 2 Hz, H-10), 6.8 (1H, d, H-9); high resolution mass spectrum, *m/e* 444.2258 (3.69%, calcd. for $C_{24}H_{32}N_2O_6$ 444.2261); 284.1885 (30.72%, calcd. for $C_{18}H_{24}N_2O$ 284.1890) 174.0918 (14.49% calcd $C_{11}H_{12}NO$ 174.0919); 124.1128 (100%, calcd. $C_8H_{14}N$ 124.1127).

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