MICROBIAL TRANSFORMATIONS OF NATURAL ANTITUMOR AGENTS. 16. CONVERSION OF LEUROSINE TO 10'-HYDROXYLEUROSINE BY *STREPTOMYCES* SPECIES

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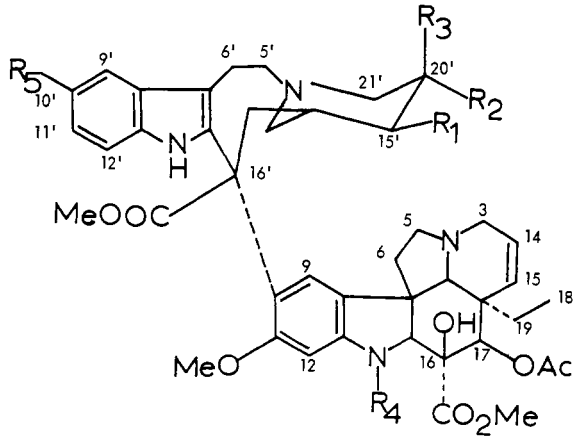
ABSTRACT.—Screening experiments with leurosine revealed its conversion to a common metabolite produced by several species of *Streptomyces*. Resting cells of *S. griseus* were employed to produce the metabolite in quantity, and spectral analysis established the structure as 10'-hydroxyleurosine.

Leurosine (**1**) is the most abundant dimeric antitumor alkaloid isolated from the Madagascan periwinkle plant *Catharanthus roseus* G. Don, and its chemical synthesis has been achieved (1–5). Leurosine is structurally related to the clinically used alkaloids vincaleukoblastine VLB (**2**) and leurocristine VCR (**3**). An investigation into the microbial metabolism of leurosine was undertaken in an effort to obtain new, active antitumor agents with lower toxicity and to uncover pathways of metabolism of dimeric Catharanthus alkaloids. This report describes the formation of a phenolic leurosine derivative (**4**) by resting cell cultures of *Streptomyces griseus* (UI 1158).



1: R_1-R_2=O R_3=Et R_4=Me R_5=H

2: R_1=H R_2=Et R_3=OH R_4=Me R_5=H

3: R_1=H R_2=Et R_3=OH R_4=CHO R_5=H

4: R_1-R_2=O R_3=Et R_4=Me R_5=OH

EXPERIMENTAL

LEUROSINE (**1**).—Leurosine sulfate was obtained from Eli Lilly and Co. and exhibited the following properties: mp 236–239 decomp; uv λ max (leurosine base) (EtOH) 263 nm (log ε 4.22),

289 (4.15), 296 (4.15); ir ν max (leurosine base) (KBr) 3459 cm^{-1}, 2918, 1730, 1608, 1220; nmr (leurosine base), δ ($CDCl_3$) 0.79, 0.92 (2t, 6H, 18-CH_3 and 18'-CH_3), 2.09 (s, 3H, 17-$OCOCH_3$), 2.70 (s, 3H, -NCH_3), 3.61 (s, 3H, 16'-CO_2CH_3), 3.79, 3.81 (2s, 6H, 16-CO_2CH_3 and 11-OCH_3), 5.28 (d, 1H, 15-H), 5.43 (s, 1H, 17-H), 5.83 (m, 1H, 14-H), 6.09 (s, 1H, 12-H), 6.57 (s, 1H, 9-H), 7.11 (m, 3H, 10',11' and 12'-H), 7.49 (d, 1H, 9'-H) and 7.97 (s, 1H, indole N-H). These data correspond well with published properties for leurosine (3, 6–10) and related alkaloids (11, 12).

GENERAL.—Infrared spectra were determined on a Beckman IR 4240 spectrophotometer and uv spectra were obtained with a Phillips Pye Unicam SP-1800 instrument. Proton nmr spectra were determined with a Bruker model WH360 instrument with $CDCl_3$ as the solvent and Me_4Si as an internal standard. Field desorption mass spectral data was obtained through Eli Lilly Research Laboratories on a Varian MAT 731 spectrometer using a direct inlet system with samples dissolved in chloroform and applied to the probe, with 15 milliamp current.

CHROMATOGRAPHY.—Thin-layer chromatography was performed on 0.25 mm layers of silica gel GF_{254} (Merck) prepared on glass plates with a Quickfit Industries spreader. Plates were air dried and oven activated for 30 minutes prior to use. Solvent systems used were: A, benzene-methanol (5:1); B, ethyl acetate-methanol-dichloromethane (1:1:1); C, chloroform-methanol (15:1). Compounds were visualized on developed chromatograms with Dragendorff reagent. Preparative thin-layer chromatography was performed on 0.5 mm layers of silica gel GF_{254} (Merck) or alumina oxide GF_{254} (E. M. Reagents) prepared as above. Solvent systems used were A (above) and D, benzene-methanol (10:1). High performance liquid chromatography (hplc) was performed on a Water Associates ALC/GPC 202 instrument equipped with a U6K universal injector and a 254 nm differential UV detector. A reversed phase Biosil ODS-10 (Bio Rad) (4 mm x 25 cm) analytical column was used with a solvent system of CH_3CN-0.005 m $(NH_4)H_2PO_4$ (70:30) at a flow rate of 1.0 ml/min.

FERMENTATION PROCEDURES.—Methods used in the cultivation of microorganisms have been described elsewhere (13). In general, a two-stage incubation procedure using a soybean meal-glucose (13) or soybean meal-glycerin medium was employed. The soybean meal-glycerine medium contained 30 g soybean meal and 20 g glycerine and distilled water to 1 liter. Incubations were conducted on rotary shakers (model G-25, New Brunswick Scientific Co.) operating at 250 rpm and 27°. The substrate, leurosine sulfate (**1**), was added to 24-hour-old stage II cultures as a solution in dimethylformamide (50 mg/ml) to a final concentration of 200 μg/ml in culture medium. Screening experiments with 96 cultures were conducted in 125 ml steel-capped DeLong culture flasks containing 25 ml of medium. Controls consisted of cultures grown without leurosine sulfate (**1**) and flasks containing leurosine sulfate in buffers at pH 3.6 (0.1M potassium phthalate), pH 6.9 (0.1M phosphate), and pH 9.5 (0.1M sodium borate). Samples were taken from controls and from substrate containing cultures at various time intervals from 1–7 days. They were adjusted to pH 9 with 28% NH_4OH and then extracted with 1 ml of ethyl acetate. Thirty μl of the ethyl acetate extracts were spotted on tlc plates.

Methods used in the preparation of resting cell incubations have been described elsewhere (14). Briefly, cultures were grown according to the usual two-stage fermentation scheme. The mycelium of the 48 hour second-stage cultures was collected by centrifugation at 8,000 xg for 15 minutes (Sorvall RC-5 refrigerated centrifuge) and was transferred to 0.1M buffer pH 6.5 (K_2HPO_4:KH_2PO_4). All cell concentrations were adjusted to correspond to 3.75 times greater than that found in growing cultures.

CONVERSION OF LEUROSINE (**1**) TO 10'-HYDROXYLEUROSINE (**4**) WITH RESTING CELLS OF *Streptomyces griseus*.—Leurosine sulfate (**1**) (400 mg) was dissolved in 3.2 ml of dimethylformamide and evenly distributed among eight 500 ml Erlenmeyer flasks containing 100 ml of cell suspensions. Substrate containing cultures were shaken for 16 hours before being harvested.

The cultures were pooled, adjusted to pH 9.0 with 28% NH_4OH and exhaustively extracted with ethyl acetate (6 liters). The extract was dried over anhydrous Na_2SO_4 and concentrated to a thick brown oil in a rotary evaporator. The residue was immediately dissolved in dichloromethane and subjected to silica gel preparative thin-layer chromatography (solvent system A). Like fractions were combined to give 69 mg of material. This was further purified by alumina preparative thin-layer chromatography (solvent system D) to yield 28.5 mg of pure alkaloid.

The metabolite possessed the following properties: Rf values on silica gel GF_{254} of 0.35, 0.48, and 0.58, in solvent systems A, B and C, respectively; retention time of 7.4 minutes (single peak) by hplc: uv, λ max (EtOH) 215 nm (log ϵ 4.63), 265 (4.10), 289 (4.08); ir ν max (KBr) 3468 cm^{-1}, 2920, 1735, 1628, 1224; nmr (Bruker model WH360) δ ($CDCl_3$) 0.88, 1.03 (2t, J=8, 8Hz, 6H, 18 and 18'CH_3) 2.18 (s, 3H, 17-$COOCH_3$), 2.79 (s, 3H, N-CH_3), 3.68 (s, 3H, 16' CO_2CH_3), 3.85, 3.87 (2S, 6H 16-CO_2CH_3 and 11-OCH_3) 5.35 (d, J=12Hz, 1H, 14-H), 5.49 (s, 1H, 17-H), 5.90 (m, 1H, 15-H), 6.14 (s, 1H, 12-H), 6.61 (s, 1H, 9-H), 6.74 (d,d, J=2, 10Hz, 1H, 11'-H), 6.90 (d, J=2Hz 1H, 9'-H), 6.98 (d, J=10Hz 1H, 12'-H), 7.83 (s, 1H, indole N-H); ms (Varian-MAT 731) M+, *m/e* 824; mass measurement observed 824.3971, calc. for $C_{46}H_{56}N_4O_{10}$ 824.3996.

RESULTS AND DISCUSSION

Microbial transformation experiments have been conducted with a wide variety of antitumor compounds including several of the *Catharanthus* alkaloids (14–18). The objectives of much of this work have been to identify novel metabolic pathways for the *Catharanthus* alkaloids and also to produce novel derivatives which may possess more favorable activities. Most of the early work was performed with vindoline, a monomeric alkaloid forming one-half of the clinically used *Catharanthus* dimers vinblastine and vincristine (14–17). Only one report to date deals with microbial transformations of the dimeric alkaloid vinblastine which yielded two metabolites identified as a VLB ether and hydroxyvinblastine (18).

Leurosine is an available dimeric *Catharanthus* alkaloid substrate with many structural features similar to those of the clinically used dimers. Screening experiments were performed with this compound, and several microorganisms were found to accumulate metabolites of the alkaloid. By tlc observation, a common metabolite was formed by *Streptomyces griseus* (UI 1158), *S. griseus* (ATCC 10137), *Streptomyces punipalis* (NRRL 3529) and *Streptomyces lavendulae* (UI 105). *Streptomyces griseus* (UI 1158) was selected for preparative scale work since it produced the metabolite consistently in apparently good yield. Resting cells of this organism were employed in the preparation of the metabolite to simplify isolation and purification of the metabolite in question. The metabolite was isolated following solvent extraction and preparative layer chromatography, but it could not be induced to crystalize as the free base. Since limited quantities of the metabolite were available, no attempt was made to form a crystalline salt.

Spectral properties of the metabolite were used to assign the structure as 10'-hydroxyleurosine (**4**). The uv and ir spectra were consistent with the retention of the dimeric indole structure of leurosine in the metabolite. While electron impact mass spectrometry failed to yield a usable spectrum, the field desorption mass spectrum revealed a strong molecular ion at *m/e* 824 for $C_{46}H_{56}N_4O_{10}$ showing the metabolite to contain one additional oxygen atom vs. leurosine itself. Nmr spectroscopy was used in identifying the position of oxygenation of the metabolite. Signals evident for protons at all portions of the molecule were identical to those displayed by leurosine itself and for analogs of leurosine previously described in the literature (12). In addition to signals observable for the aromatic protons of vindoline at 6.09 and 6.57 ppm, the spectrum of leurosine contains a multiplet at 7.11 ppm corresponding to signals for aromatic protons 10', 11' and 12', and also a doublet at 7.49 ppm for the proton at position 9'. These assignments are analogous to those published for 21'-hydroxyleuosine and 21'-oxoleurosine (12) and are consistent with assignments for aromatic protons on simple substituted indoles as well. The nmr spectrum of the metabolite contained signals for the aromatic vindoline protons, but signals for aromatic protons of the Iboga portion of the molecule occurred as a doublet of doublets at 6.74 ppm ($J=2$, 10 Hz, 1H), a doublet at 6.90 ppm ($J=2$Hz, 1H), and a doublet at 6.98 ppm ($J=10$ Hz, 1H). These signals are consistent with the presence of an Ha, Hm, Hx system and indicate an arrangement of protons with a hydroxyl group attached at either position 10'- or 11' of the indole ring of leurosine. The hydroxyl group is assigned to position 10' based on the magnitude of the upfield shift of the proton signal at position 9' from 7.49 ppm in leurosine to 6.90 ppm in the metabolite. The equivalent proton signals for 10'-hydroxyvinblastine (18), 9-hydroxyellipticine (19), and 5-methoxyindole (20) experience shielding effects of 0.50, 0.50 and 0.41 ppm,

respectively, vs. the unsubstituted indole systems. In contrast, substitution of the indole ring system with oxygen at position 6 for indole itself (20) and position 8 of ellipticine (19), for example, results in little (0.1 ppm) or no change in the position of the same proton signal. It is noteworthy that similar arguments were used in assigning the structure of the known 10'-hydroxyvinblastine where nearly identical patterns of aromatic proton signals were reported (18).

Neuss *et al.* reported an analogous microbial conversion reaction using vinblastine which was converted into the equivalent phenolic hydroxyvinblastine (18). Other examples of microbial aromatic hydroxylation of alkaloids like acronycine and ellipticine have been observed (13, 19, 21). In general, the microbial hydroxylation of aromatics results in the formation of products predictable by the usual rules of electrophilic aromatic substitution (21) where hydroxylations occur at positions *ortho-* or *para-* to ring activating substituents such as nitrogen or oxygen. On this basis, the formation of 10'-hydroxyleurosine (**4**) finds precedence in other microbial aromatic hydroxylations. Further studies with leurosine are being conducted in order to isolate and identify minor metabolites formed by Streptomyces.

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