MICROBIAL TRANSFORMATIONS OF NATURAL ANTITUMOR AGENTS. 9. O-DEMETHYLATION OF 9-METHOXYELLIPTICINE

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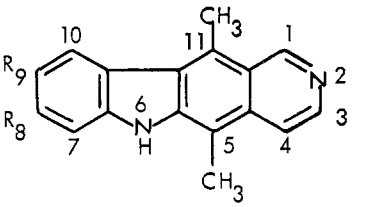
Abstract.—Microbial transformation studies were conducted with 9-methoxyellipticine (**1**). Several microorganisms including *Botrytis allii* (NRRL 2502), *Cunninghamella echinulata* (NRRL 1386), *C. echinulata* (NRRL 3655), and *Penicillium brevicompactum* (ATCC 10418) achieved O-demethylation of (**1**) in good yield. *P. brevicompactum* was used to prepare 9-hydroxyellipticine (**4**) from **1** for isolation and complete identification. High-performance liquid chromatography was used to verify the identity of the major metabolite (**4**) in other cultures.

9-Methoxyellipticine (**1**) is an antitumor pyridocarbazole alkaloid which was isolated from *Aspidosperma vargasii* (1), *Bleekeria vitiensis* (2) and many species of *Ochrosia* (3–5). This alkaloid often coexists with the parent alkaloid ellipticine (**2**) (2, 3, 5), and both compounds display significant antitumor activity against several experimental neoplasms (6, 7). The chemical synthesis of 9-methoxyellipticine has been achieved (8–10).



1 R = H, $R_9 = OCH_3$
2 $R_8 = R_9 = H$
3 $R_8 = OH$, $R_9 = H$
4 $R_8 = H$, $R_9 = OH$

Fig. 1. The structures of various ellipticine derivatives.

We are employing microorganisms as useful tools in studying the metabolism of a variety of antitumor compounds (11). In a previous report (12), we described the hydroxylation of ellipticine by *Aspergillus alliaceus* to 8- and 9-hydroxyellipticines (**3** and **4**). The latter compound is also a major mammalian metabolite of ellipticine (13–16). This report is the first to describe microbial transformation studies with 9-methoxyellipticine (**1**).

EXPERIMENTAL[1]

[1]Melting points were determined in open-ended capillary tubes in a Thomas-Hoover capillary melting point apparatus and were corrected. Ir spectra were obtained on a Perkin Elmer 267 spectrophotometer with KBr discs. Uv spectra were recorded in a Beckman Ratio Recording spectrophotometer. Nmr spectra were recorded with a Varian T-60 spectrometer with tetramethylsilane as internal standard and chemical shifts reported in δ (ppm) units. The mass spectra were obtained with a Finnigan model 3200 spectrometer.

9-METHOXYELLIPTICINE (**1**).—9-Methoxyellipticine (NSC No. 69187) was obtained from the National Cancer Institute and was fully characterized as follows: mp 260° (decomp.); uv, λmax (MeOH) 211 nm (log ε 4.23), 245 (4.31), 276 (4.55), 291 (4.62), 337 (3.71), 354 (3.45) and 403 (3.44); ir, νmax (KBr) 3150 cm^{-1}, 1600, 1470, 1380, 1295, 1250, 1220, 1140, 1020 and 810; nmr, δ (DMSO-d_6) 2.67 (s, 3H, C_{11}-CH_3), 3.13 (s, 3H, C_5-CH_3), 3.82 (s, 3H, C_9-OCH_3), 7.03 (dd, J=2,8Hz, C_8-H), 7.33 (d, J=8Hz, C_7-H), 7.66 (d, J=2Hz, C_{10}-H), 7.67 (d, J=6Hz, C_3-H), 8.20 (d, J=6Hz, C_4-H), 9.28 (s, C_1-H) and 10.72 (s, NH); ms, M^+, *m/e* 276 (83%), 261 (100), 233 (24), 218 (14), 204 (9), 190 (9), 138 (38), 116 (28), 102 (25), 96 (11), 88 (15) and 76 (12). These data correspond well with published properties for the alkaloid (1, 4, 5).

CHROMATOGRAPHY.—Thin-layer chromatography was performed on 0.25 mm-thick layers of silica gel GF_{254} (Merck) prepared on glass plates with a Quickfit Industries spreader. Plates were air dried and then oven activated at 120° for 30 min prior to use. Solvent systems used in developing tlc plates were: A, chloroform-ethanol-acetic acid (70:30:2); B, ethyl acetate-ethanol-diethylamine (95:5:1); and C, chloroform-methanol (4:1). Compounds were detected on developed chromatograms by fluorescence quenching under 254 or 365 nm uv irradiation and were later visualized by spraying with Dragendorff reagent. Column chromatography was performed using silica gel (Baker 3405) which was activated in an oven at 120° for 60 min prior to use. Columns were slurry-packed in the developing solvent, and fractions were collected with a Fractomette 200 instrument or manually.

High performance liquid chromatography (hplc) was performed using 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. Ellipticines were well separated on reversed phase μBondapak-Phenyl or μBondapak-C_{18} analtyical columns (Waters) (3.9 mm x 30 cm). The hplc properties of **1** and **4** are recorded in table 1.

TABLE 1. Hplc properties of 9-hydroxyellipticine and 9-methoxyellipticine.

	Retention Volume (ml)[3]		
	μBondapak C_{18} column with MeOH:0.1% $(NH_4)_2CO_3$	μBondapak/Phenyl with CH_3CN:0.1% $(NH_4)CO_3$	
	(3:2)	(2:3)	(55:45)
9-Hydroxyellipticine.....	11.2	16.8	6.4
9-Methoxyellipticine.....	27.0	40.0	10.2

[3]Analyses were performed at 1000 p.s.i. with flow rate 1.0 ml/min or 1600 p.s.i. with flow rate 1.4 ml/min.

Hplc analyses of cultures containing 9-methoxyellipticine at a concentration of 400 μg/ml of culture medium were performed as follows. After an incubation period of 6 days, cultures were adjusted to pH 8.5 with ammonium hydroxide and extracted with an equal volume (25 ml) of ethyl acetate-*n*-butenol (9:1). Extracts were clarified by centrifugation at 10,000 x g (Sorvall RC5 centrifuge), and 10 μl volumes of the extracts were injected for analysis. Recoveries of 9-methoxyellipticine and 9-hydroxyellipticine from aqueous media averaged 90% by this procedure.

FERMENTATION PROCEDURE.—Methods used in the cultivation of microorganisms have been described elsewhere (17). In general, a two-stage incubation procedure using a soybean meal-glucose medium was employed. Incubations were conducted on rotary shakers (Model G-25 New Brunswick Scientific Co.) operating at 250 rpm and 27°. The substrate, 9-methoxyellipticine (**1**), was added to 24-hour-old stage II cultures as a solution in dimethylformamide to a final concentration of 400 μg/ml of culture medium. Screening experiments with 211 cultures were conducted in 125 ml steel-capped Delong culture flasks holding 25 ml of medium. Controls consisted of cultures grown without 9-methoxyellipticine (**1**) and of flasks containing buffers at pH 3.0 (0.1M citrate-phosphate), pH 6.8 (.066M phosphate) and pH 8.5 (0.2M Tris) without microorganisms. Samples (4 ml) were taken from controls and from substrate-containing cultures at various time intervals from 1–7 days. They were adjusted to pH 8 with ammonium hydroxide solution and then extracted with 1 ml of ethyl acetate-*n*-butanol (9:1). Thirty μl of the extracts were spotted on tlc plates.

CONVERSION OF 9-METHOXYELLIPTICINE (**1**) TO 9-HYDROXYELLIPTICINE (**4**) WITH *Penicillium brevi-compactum*. 9-Methoxyellipticine (**1**) (400 mg) was dissolved in 4 ml of dimethylforma-

mide and evenly distributed among 10 one-liter steel-capped Delong culture flasks, each holding 200 ml of 24-hour Stage II cultures of *P. brevi-compactum*. Cultures were harvested by filtration after 6 days of incubation, and cells and culture filtrates were treated separately.

Cells were macerated in ethyl acetate-*n*-butanol (9:1) (500 ml x 2), filtered, and then extracted with methanol (500 ml x 2). The methanol extract (2.624 g) was purified with a silica gel column (100 g, 3 x 33 cm) which was eluted first with chloroform-methanol (19:1) (1 liter) at a flow rate of 5 ml/min to give 0.103 g of **1**. Further elution of the column with 9:1 mixtures of the same solvent (0.3 liter) gave 0.07 g of impure **4**.

The culture filtrate was adjusted to pH 8.5 with 10% ammonium hydroxide and exhaustively extracted with ethyl acetate-*n*-butanol (9:1) (6 liters); the extract was dried over anhydrous sodium sulfate and concentrated at 40° in a rotary evaporator.

Ethyl acetate-*n*-butanol extracts from cells and filtrates were similar in composition and were combined. This extract (0.951 g) was purified by a silica gel column (100 g, 3 x 33 cm) eluted with chloroform-methanol mixtures of increasing polarity at a flow rate of 5 ml/min while fractions of 50 ml were collected. 9-Methoxyellipticine (**1**) (0.171 g) was recovered following elution with chloroform-methanol (19:1) (1 liter); nearly pure 9-hydroxyellipticine (**4**) (0.157 g) was obtained after further elution with (9:1) mixtures of the same eluant (2 liters). All of the 9-methoxyellipticine recovered from both columns was combined and crystalized from hot methanol as yellow needles (0.2 g).

All fractions containing the metabolite (**4**) were combined and finally purified with an alumina column (80 g, 1.5 x 35 cm) eluted with chloroform-methanol (4:1) at a flow rate of 4 ml/min. Fractions of 50 ml were collected. The pure metabolite was obtained from fractions 2–5 as a yellow solid which crystallized as fine yellow needles from hot chloroform-methanol (1:1) mixtures (0.15 g). It displayed the following properties: tlc, R_f values of 0.32, 0.48 and 0.32 in solvent systems A, B and C respectively; mp 280° (decomp.); uv, λmax (MeOH), 245 nm (log ε 4.41), 276 (4.60), 292 (4.64), 336 (3.84), 353 (3.45) and 405 (3.44); λmax in 0.1 *N* methanolic KOH, 249 (4.41), 277 (4.55), 292 (4.59), 353 (3.52), 368 (3.40) and 415 (3.36); ir, νmax (KBr) 3270 cm^{-1}, 1600, 1580, 1470, 1400, 1260, 1215, 1140, 1030 and 810; nmr δ(DMSO-d_6) 2.73 (s, 3H), 3.20 (s, 3H), 6.95 (dd, J=2, 8Hz), 7.28 (d, J=8Hz), 7.70 (d, J=2Hz), 7.78 (d, J=6Hz), 8.30 (d, J=6Hz), 9.37(s) and 10.65(s); ms, M^+, *m/e* 262 (100%), 247 (37), 233 (9), 231 (8), 218 (11), 204 (10), 191 (10), 190 (10), 176 (8), 164 (9), 151 (11), 131 (51), 122 (10), 117 (20), 116 (20), 109 (19), 102 (33), 96 (24), 88 (23), 76 (16) and 63 (14). The microbial metabolite was comparable in all respects to synthetic 9-hydroxyellipticine (12). Comparisons included melting point, mixture melting point (no depression), ir, nmr, uv, ms and co-chromatography on tlc and hplc.

RESULTS AND DISCUSSION

Screening scale experiments revealed several cultures with the ability to accumulate 9-methoxyellipticine metabolites. By tlc observation, nearly half of the substrate was converted to a common major metabolite with *Botrytis allii* (NRRL 2502), *Cunninghamella echinulata* (NRRL 1386) and *P. brevi-compactum* (ATCC 10418). A preparative scale incubation using *P. brevi-compactum* with 0.4 g of **1** resulted in the production of 0.15 g of the metabolite; 200 mg of unreacted substrate was recovered.

Spectral properties of the metabolite indicated that it was 9-hydroxyellipticine (**4**). The methoxyl protons of **1** (3.82 ppm) were absent in the nmr spectrum of the metabolite, while a singlet (8.78 ppm) could be assigned to a phenolic proton. All other proton signals for **1** and the metabolite were comparable. The molecular ion (*m/e* 262) of the metabolite also suggests loss of the methyl ether, and the phenolic hydroxyl was confirmed by the bathochromic shift of its uv spectrum observed in basic medium. Comparison of ir, uv, mass spectral, mp and chromatographic properties of the isolated metabolite with authentic 9-hydroxyellipticine confirmed its identity as **4**.

Hplc was used to verify the identity of 9-hydroxyellipticine as a microbial metabolite of **1** in other cultures. This approach was used in order to remove the possibility that other metabolites with similar tlc mobilities might be obtained (12). Several types of columns and solvent systems were initially examined for their abilities to separate 9-methoxyellipticine (**1**) and 9-hydroxyellipticine (**4**) as well as other ellipticine derivatives. Reversed-phase systems of μBondapak-C_{18}

(16) and μBondapak-phenyl columns (Waters) used with eluants of methanol-0.1% ammonium carbonate (3:2) and acetonitrite-0.1% ammonium carbonate (2:3), respectively, achieved excellent separations of test compounds. When extracts of several cultures were subjected to hplc analysis, the following yields of 9-hydroxyellipticine were obtained: *Basidiololus varnarum* UI-BV (10%); *Botrytis allii* NRRL 2502 (36%); *Cunninghamella echinulata* NRRL 1386 (39%); *C. echinulata* NRRL 3655 (25%); *C. elegans* NRRL 1393 (8%) and *Penicillium brevicompactum* ATCC 10418 (34%). It is noteworthy that these cultures were all harvested at the same time (6 days following substrate addition) and that fermentations were not optimized to improve yields.

O-Demethylation is a common microbial metabolic transformation of 9-methoxyellipticine, and yields of metabolites are high. Many other examples of microbial *O*-demethylation may be cited to show that this fermentation-type reaction occurs in good yield and that it may be used to accomplish selective *O*-demethylation even with substrates not amenable to chemical demethylation methods. These include regioselective *O*-demethylations of griseofulvin (18), 10,11-dimethoxyaporphine (19), papaverine (20) and glaucine (21); and demethylation of vanillic acid (22), 7-methoxyisoflavone (23), aspidospermin (24) and vindoline (25). This is the first report on the microbial metabolism of 9-methoxyellipticine. The major microbial (12) and mammalian (13–16) metabolite of elelipticine is 9-hydroxyellipticine, and it will be interesting to learn whether *O*-demethylation of **1** will also be a major mammalian transformation reaction. This will be important because 9-hydroxyellipticine has the highest degree of antineoplastic activity among the known ellipticines (26, 27).

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