Microbial Metabolites of Harman Alkaloids

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Several microorganisms showed the ability to transform the harman alkaloids, harmaline (1), harmalol (2) and harman (5). Harmaline (1) and harmalol (2) were converted by *Rhodotorula rubra* ATCC 20129 into the tryptamines, 2-acetyl-3-(2-acetamidoethyl)-7-methoxyindole (3) and 2-acetyl-3-(2-acetamidoethyl)-7-hydroxyindole (4), respectively. Harman (5) was biotransformed by *Cunninghamella echinulata* NRRL 3655 into 6-hydroxyharman (6) and harman-2-oxide (7).

Key words harman; harmaline; harmalol; microbial; metabolites; NMR

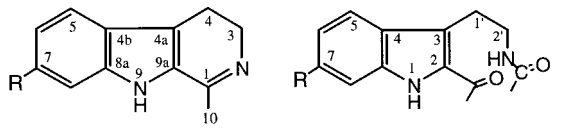
Harman alkaloids are important constituents of the medicinal plant, *Perganum harmala* (Syrial rue) belonging to the family Zygophylaceae. In traditional medicine, this plant is used to treat asthma, jaundice, lumbago and many other human ailments[1,2] The seeds of the plant constitute the most concentrated natural source of harmaline (**1**) and harmine. Harmaline is a known inhibitor of monoamine oxidase, a potent serotonin antagonist and a hallucinogen[3] A considerable amount of biological activity studies has been carried out on harmaline and its derivatives.[4,5] Harman has been detected in several plant species[6] including *Passiflora incarnata*, which is used as a dietary supplement. It is also present in grape juice and wine.[7] The detection of harman in well-cooked foods, charred insects, tobacco smoke and marijuana smoke, suggested its formation from tryptophan by pyrolysis.[6] It has been identified in human platelets,[8] while experiments have indicated that ethanol induces an increase of harman levels in the brain and urine in rats.[9]

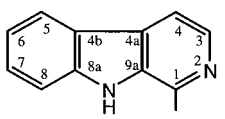
Investigations into the metabolism of harmaline (**1**) in rats have revealed that it crosses the blood-brain barrier readily and acts directly on the central nervous system.[10] Studies have also revealed that the major fate of this compound was demethylation to form harmalol (**2**), which gets excreted as the glucuronide conjugate.[11] In addition, about 10% of harmalol undergoes dehydrogenation to yield harmol, which subsequently gets excreted as the sulphate conjugate. Harmalol brings about progressive paralysis of the central nervous system.[11] Apart from the metabolites in the urine, no chemical analysis of the transformed products formed in tissues has been reported. Therefore, a microbial transformation study was undertaken to generate the mammalian metabolites of **1**, **2** as well as **5**. Microbial metabolism often parallels that of mammals and has been successfully used to mimic mammalian drug metabolism.[12,13] The formation of sufficient quantities of metabolites in such studies often facilitates structure elucidations and biological investigations.[13]

As part of our program on microbial transformation studies, the harmala alkaloids, harmaline (**1**), harmalol (**2**) and harman (**5**) were screened using thirty-seven microorganisms. The standard two-stage fermentation procedure[14] was adopted with suitable controls to ensure that the compounds obtained were due to enzymatic activity. TLC analysis of the culture extracts revealed that several microorganisms were capable of converting the above alkaloids (Table 1).

Rhodotorula rubra, was selected for preparative-scale studies on harmaline **1** and harmalol **2**, due to its high efficiency of bioconversion.

Preparative scale fermentation of harmalol (**2**) (300 mg) with *R. rubra*, gave 2-acetyl-3-(2-acetamidoethyl)-7-hydroxyindole (**4**) (30 mg, 7.6%). The exact molecular mass (*m*/*z* 260.0453; M^+) of **4** was consistent with the molecular for-





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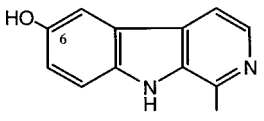

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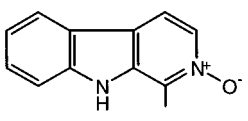

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Table 1.

Compound	Organisms	
Harmaline	*Rhodotorula rubra*	ATCC 20129
	Pichia membranifaciens	ATCC 2254
	Steptomyces rimosus	ATCC 10970
	S. griseus	ATCC 10137
	S. lavendulae	ATCC 8664
	Lodderomyces elongospores	ATCC 22688
Harmalol	*Rhodotorula rubra*	ATCC 20129
Harman	*Streptomyces rimosus*	ATCC 10970
	Mucor ramannianus	ATCC 9628
	Cunninghamella species	NRRL 5695
	C. elegans	ATCC 9245
	C. elegans	ATCC 10028b
	C. echinulata	NRRL 3655
	C. echinulata	ATCC 9244
	Ramichloridium anceps	ATCC 15672
	Streptomyces roseochromgenus	ATCC 13400
	Chaetomium cochloides	ATCC 10195
	Beauveria bassiana	ATCC 13144
	Aspergillus flavipes	ATCC 1030

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mula, $C_{14}H_{16}N_2O_3$. The ^{1}H-NMR spectrum of **4** differed from that of **1** in the disappearance of the three-proton singlet at δ 2.73 (C–Me) and the appearance of two sharp three-proton singlets at δ 2.57 (Ac) and 1.90 (–NAc). The ^{13}C-NMR spectrum accordingly showed two carbonyl signals at δ 191.94 (Ar<u>C</u>O) and 172.33 (–N<u>C</u>O) and two high field methyl resonances at δ 27.97 (ArCO<u>C</u>H$_3$) and δ 22.53 (–NCO<u>C</u>H$_3$). The peak at δ 18.4 due to C-14, observed in harmalol (**2**), was absent in **4**. Compound **4** was thus identified as the new tryptamine derivative, 2-acetyl-3-(2-acetamidoethyl)-7-hydroxyindole. The ^{1}H and ^{13}C data collated in Table 2 were fully corroborated by appropriate two dimensional (2D) NMR experiments, *e.g.* correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC).

Preparative scale fermentation of harmaline (**1**) (300 mg) by *R. rubra* yielded 2-acetyl-3-(2-acetamidoethyl)-7-methoxyindole (**3**) (110 mg, 28.6%). High-resolution mass spectroscopic analysis of **3** (*m*/*z* 274.1317; M^+) indicated a molecular formula of $C_{15}H_{18}N_2O_3$. The metabolite was identified as the tryptamine derivative, 2-acetyl-3-(2-acetamidoethyl)-7-methoxyindole, based on spectral data (Table 2). The reasoning for structure elucidation was the same as for metabolite, **4**. This compound has been previously prepared chemically from harmaline and tested for biological activity.[5] The study revealed that **3** exhibited increased activity against several Gram-positive bacteria and a decrease in toxicity, compared to harmaline (**1**). Experiments on the effects of harmaline (**1**) on the central nervous system of mice showed intense tremor and subsequent death. Similar experiments with **3** indicated no changes in the gross behavior of animals.[5] Tryptamines **3** and **4** may be used as analytical standards for detection and characterization of the metabolites of harmaline (**1**) and harmalol (**2**) in biological fluids. These can also be used for further pharmacological investigations.

Table 2. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) Data for Tryptamines **3** and **4** in $CDCl_3$

Position	**3**		**4**	
	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)
2	131.9	—	131.6	—
3	121.9	—	122.0	—
4	138.3	—	138.7	—
5	122.1	7.45 d (8.8)	122.6	7.47 d (8.7)
6	112.7	6.71 dd (8.8, 1.6)	112.6	6.69 dd (8.7, 1.9)
7	160.1	—	157.3	—
8	94.1	6.74 d (1.6)	96.5	6.75 d (1.9)
9	138.3	—	138.8	—
1′	25.2	3.21 t (6.7)	25.0	3.24 t (6.9)
2′	41.4	3.46 t (6.7)	41.3	3.46 t (6.9)
OCH_3	55.8	3.74 s	—	—
Ar–CO<u>C</u>H$_3$	28.3	2.53 s	28.0	2.57 s
Ar–<u>C</u>O–	191.4	—	191.9	—
N–CO<u>C</u>H$_3$	23.2	1.87	22.5	1.90
N–CO–	171.6	—	172.3	—

Cytochrome P-450-dependent mono-oxygenases could catalyze the conversion of harmaline (**1**) and harmalol (**2**) into their respective tryptamines, **3** and **4** (Chart 1). Thus, epoxidation of the imino functionality would give oxaziridine **8**, which could be transformed into **9** *via* hydrolysis. Reaction with acetyl CoA would then afford the *N*-acyl hydroxylamine **10**, which may be hydrolyzed to give imine **11**. Reduction of the imino group would then afford the tryptamines **3** and **4**. Alternatively, the imine functionality in **1** and **2** may be cleaved hydrolytically, followed by a concerted process of acetylation.

Screening-scale studies indicated that twelve of the thirty-seven microorganisms were capable of transforming harman **5** into two metabolites (Table 1). *Cunninghamella echinulata* NRRL 3655 was selected for preparative-scale fermentation studies, as it showed complete conversion of the alkaloid. The ethyl acetate extract of the medium of 14 d old culture of Harman **5** (300 mg), when subjected to repeated column chromatography yielded two metabolites, **6** (21.4 mg, 6.5%), **7** (8.7 mg, 2.7%). The high-resolution MS data of both metabolites were consistent with a molecular formula of $C_{12}H_{10}N_2O$. The additional oxygen in **6** was present as a phenolic hydroxyl group as determined by IR, ^{1}H- and ^{13}C-NMR data. The location of the hydroxyl group was determined by 2D NMR experiments. A direct comparison of this metabolite with 6-hydroxyharman (IR, NMR)[15] gave further evidence for its structure. The ^{1}H- and ^{13}C-NMR together with 2D NMR data of **7** indicated that it was harman-2-oxide, which has been previously reported from *Ophiorrhiza rocaceae*.[16] It has also been found in wine. Its structure was further confirmed by comparing (UV, IR and NMR) with the synthetic *N*-oxide formed by oxidizing harman with *m*-chloroperbenzoic acid. 6-Hydroxyharman (**6**) and harman *N*-oxide (**7**) are more polar than harman and may contribute to the elimination of the parent compound. 6-Hydroxyharman is also the major metabolite formed when harman is treated with mice microsomal preparations.[17]

The major harman alkaloids are thus subject to facile microbial transformation into metabolites which may provide

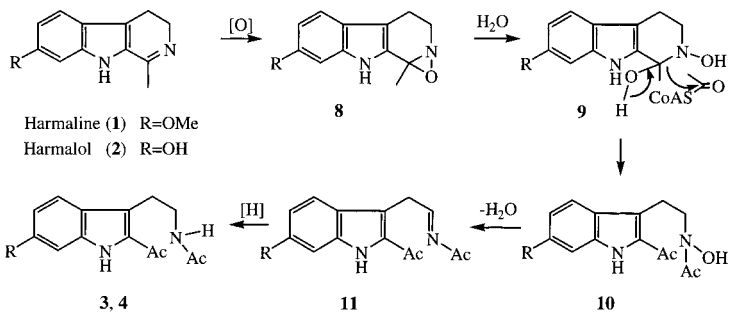

Chart 1. Proposed Mechanism of the Cytochrome P-450-Dependent Mono-Oxygenation of Harmaline and Harmalol

useful information regarding the metabolism of drugs in mammalian systems.

Experimental

General Experimental Procedure Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. The IR spectra were run in $CHCl_3$ using an ATI Mattson Genesis Series FTIR Spectrophotometer. The ^{1}H- and ^{13}C-NMR were recorded in $CDCl_3$ on a Bruker Avance DRX-500 FT spectrometer. High resolution electrospray ionization mass spectroscopy (HR-ESI-MS) data were obtained using a Bruker GioApex 3.0 apparatus.

Substrates Harman, harmaline and harmalol were from Aldrich (Milwaukee, U.S.A.). The authenticity of the alkaloids was established by physical methods including NMR and HPLC.

Organisms and Metabolism Thirty-seven microorganisms obtained from the National Center for Natural Products Research, University of Mississippi were used for screening. All the fermentation experiments were carried out in medium α, consisting of dextrose, 20 g; NaCl, 5 g; K_2HPO_4, 5 g; bacto-peptone (Difco Labs), 5 g and yeast extract (Difco Labs, Detroit, MI, U.S.A.), 5 g per liter of distilled water. Initial fermentations were conducted in 125 ml Erlenmeyer flasks containing 25 ml medium α. A two-stage fermentation procedure was adopted in all experiments.[14] Each substrate was added in dimethylformamide (0.5 mg/ml) to 24 h old stage II cultures. They were incubated at room temperature on a rotary shaker (New Brunswick Model G10-21) at 100 rpm for a period 14 d. Sampling and TLC monitoring were carried out at 7 d intervals. Precoated Si gel 60 F_{254} plates (E. Merck) were used with $CHCl_3 : (CH_3)_2CO : Et_2NH$ (5 : 4 : 1) as the solvent system. UV light (254 nm) and *p*-anisaldehyde spray reagent were used to visualize the spots. Scale-up fermentations were performed under the same conditions with six 2 l flasks, containing 300 ml of medium and 50 mg of substrate, each. Extraction of the culture filtrates and residues was carried out with EtOAc. The solvent was evaporated *in vacuo* at 40 °C to obtain the residues. The purification of metabolites was carried out by column (Si gel 230—400 mesh: E. Merck) and preparative layer (Si gel 60 F_{254}) chromatography. Culture and substrate controls were run simultaneously with the above experiments.[18]

Microbial Transformation of Harmaline (1) by *R. rubra* The incubation mixtures of harmaline (**1**) (300 mg) were pooled and filtered. The filtrate (1.8 l) was extracted exhaustively with EtOAc and a brownish solid (941 mg) was obtained on evaporation of the solvent. It was column chromatographed over silica gel with $CHCl_3$ gradually enriched with MeOH as the eluting solvent. 2-Acetyl-3-(2-acetamidoethyl)-7-methoxyindole, **3** was crystallized from $CHCl_3$, to yield while needles (110 mg, 28.6% yield), mp 162 °C (lit.[5] mp 160—162 °C; *Rf* 0.64; UV (MeOH) λ_{max} (log ε): 218 (3.88), 258 (3.38), 336 (3.86) nm; IR ν_{max} (CHCl3) cm^{-1}: 3287, 3092, 2933, 1640, 1630, 1573, 1529, 1428, 1161; ^{1}H- and ^{13}C-NMR: see Table 2; HR-ESI-MS *m/z* $[M+Na]^+$: 297.1196 (Calcd for $C_{15}H_{18}N_2O_3$+Na: 297.12092).

Microbial Transformation of Harmalol (2) by *R. rubra* ATCC 20129 The procedures for fermentation of harmalol **2** (300 mg), extraction and separation of the new tryptamine, 2-acetyl-3-(2-acetamidoethyl)-7-hydroxyindole (**4**) were as described for harmaline. The EtOAc extract of the filtrate on separation gave a white solid (30 mg, 7.6%); *Rf* 0.79; UV (MeOH) λ_{max} (log ε): 220 (3.75), 258 (3.34), 336 (3.85) nm; IR ν_{max} ($CHCl_3$) cm^{-1}: 3290, 2933, 1624, 1572, 1530, 1430; ^{1}H- and ^{13}C-NMR: see Table 2; HR-ESI-MS *m/z* $[M+Na]^+$: 283.1053 (Calcd for $C_{14}H_{16}N_2O_3$+Na: 283.1061).

Microbial Transformation of Harman (5) by *C. echinulata* NRRL 3665 The EtOAc extract (773.2 mg) of the culture medium (300 mg of **5**) when subjected to column chromatography with $CHCl_3$ enriched with MeOH yielded 6-hydroxyharman (**6**) (21.4 mg, 6.5%) and harman-2-oxide (**7**) (8.7 mg, 2.7%). Compound (**6**) gave yellow needles from MeOH, mp 319 320 °C; *Rf* 0.31; UV (MeOH) λ_{max} (log ε): 234 (4.35), 246 (4.28), 258 (4.15), 288 (3.98), 296 (4.16) 360 (3.60) nm; IR ν_{max} (KBr) cm^{-1}: 3207, 1577, 1458, 1199, 1027, 817; ^{1}H-NMR (500 MHz, CD_3OD): δ 8.07 (1H, d, *J*=5.4, H-3), 7.76 (1H, d, *J*=5.4, H-4), 7.48 (1H, d, *J*=2.2, H-5), 7.42 (1H, *J*=8.7, H-8), 7.12 (1H, dd, *J*=8.7, 2.2, H-7), 2.76 (3H, s, Me-1). ^{13}C-NMR: δ 151.2 (C-6), 142.0 (C-1), 136.0 (C-3), 135.9 (C-4b), 128.3 (C-4a), 122.3 (H-8a), 118.4 (C-7), 113.0 (C-4), 112.5 (C-8), 105.7 (H-5), 18.6 (Me-1); HR-ESI-MS *m/z* $[M+H]^+$: 199.0865 (Calcd for $C_{12}H_{10}N_2O$+H: 199.0866). Compound **7** was amorphous; *Rf* 0.23; UV (MeOH) λ_{max}(log ε): 256 (4.44), 316 (4.28), 344 (3.68), 358 (3.54) nm; IR ν_{max} (KBr) cm^{-1}: 3353, 3195, 1618, 1455, 1382, 1204, 1171, 1094, 762; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.07 (1H, d, *J*=6.4, H-3), 7.93 (1H, d, *J*=6.7, H-5), 7.78 (1H, d, *J*=6.4, H-4), 7.49 (1H, dd, *J*=7.2, 7.5, H-7), 7.45 (1H, d, 7.5, H-8), 7.21 (1H, dd, *J*=6.7, 7.2, H-6), 2.71 (3H, s, Me-1). ^{13}C-NMR: δ 142.54 (C-8a), 135.96 (C-8b), 134.60 (C-1), 130.58 (C-3), 128.30 (C-7), 122.77 (C-4a), 121.27 (C-4b), 121.14 (C-5), 120.69 (C-6), 114.22 (C-4), 111.80 (C-8), 11.71 (Me-1) HR-ESI-MS *m/z* $[M+H]^+$: 199.0876 (Calcd for $C_{12}H_{10}N_2O$+H: 199.0866).

Preparation of Harman-2-oxide 7 A mixture of harman (**5**) (100 mg) and *m*-chloroperbenzoic acid (100 mg) in $CHCl_3$ was allowed to stand at room temperature for 24 h. It was washed with 5% $NaHCO_3$ solution. The organic layer was evaporated and the residue was chromatographed over Si gel to yield harman-2-oxide (**7**) as a white solid (18.60 mg, 17.2%) with physical data identical to those of the product from microbial transformation.

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