Microbial Biotransformation of Veratramine

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Preparative-scale fermentation of the known C-*nor*-D-homosteroidal alkaloid veratramine (**1**) with *Nocardia* species ATCC 21145 has resulted in the isolation of three new metabolites, **2**−**4**. The structure elucidation of these compounds was conducted primarily by 2D NMR analysis. The microbe *Nocardia* species ATCC 21145 was able to metabolize rings A and B of veratramine but failed to metabolize its nitrogen-containing side chain, an observation consistent with previous fermentation studies on steroidal alkaloids.

Veratrum alkaloids represent a group of potent hypotensive agents that lower blood pressure by reflex suppression of the cardiovascular system.[1] Veratramine (**1**) is a known veratranine C-*nor*-D-homosteroidal *Veratrum* alkaloid.[2] It antagonizes the Na^+ channel-gating mechanism of ceveratrum alkaloids by blocking the Na^+ channels.[3] Veratramine also shows serotonin (5-HT) agonist activity, acting on presynaptic 5-HT neurons. The administration of vertatramine induces generalized tremors, myoclonus, hindlimb abduction, backward gait, and Straub tail, similar to the 5-HT syndrome in mice.[4] Recently, **1** was reported to induce hemolysis of human red blood cells.[5]

Metabolism studies have been used successfully as model systems to predict metabolic pathways in humans or to increase the efficacy of drugs by metabolic activation. Microorganisms have recently been used as *in vitro* models for predicting mammalian drug metabolism.[6] Steroidal alkaloids have been investigated in metabolism studies due to their interesting biological activities.[7] Tomatidine is a known spirosolane-type *Solanum* alkaloid with the commonly encountered six-membered C-ring and five-membered D-ring steroidal skeleton. Incubation with *Nocardia restrictus*, *Mycobacterium phlei*, and *Gymnoascus reesii* resulted in the metabolism of tomatidine to 1,4-tomatidien-3-one, tomatidone, tomatanin-3α-ol, 1-tomatiden-3-one, and 4-tomatiden-3-one but failed to induce any *N*-containing side-chain modification.[7,8] The only previous attempt to study the microbial metabolism of a C-*nor*-D-homosteroidal alkaloid enabled the conversion of jervine into an unidentified antibacterial product using *Piricularia oryzae*.[9] In the present study, veratramine (**1**) was chosen for a microbial bioconversion study in an attempt to prepare less toxic and more bioactive analogues and to check whether there would be any metabolism in the *N*-containing side chain.

Twenty-five growing cultures were screened for their ability to bioconvert **1**. Few were observed to transform **1** completely or partially to metabolites of the same or greater polarity. *Nocardia* species ATCC 21145 was chosen for preparative-scale fermentation because it entirely depleted **1** and converted it into a compound of the same polarity, **2**, and two more polar ones, **3** and **4**.

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The FT-IR spectrum of **2** showed a strong absorption band at 1705 cm^{-1}, indicating the presence of a ketone. The HRFABMS data of **2** suggested a molecular formula of $C_{27}H_{39}O_2N$. Comparison of the ^1H- and ^{13}C-NMR data of **2** (Table 1) with those published for **1**,[10] suggested the disappearance of the Δ^5 system and the hydroxyl group at C-3, and the appearance of a new carbonyl group, which was observed at 211.4 ppm. The chemical shift value of the C-19 methyl group (11.6 ppm) indicated that the H-5 proton was in the α-orientation.[2] The ^{13}C-NMR data of rings A and B indicated that the A−B ring junction was *trans* and further supported structure **2**.[11] Accordingly, metabolite **2** was assigned as (23*R*)-12,13,14,15,16,17-hexadehydro-23-hydroxy-5α-veratranin-3-one.

The UV absorption maximum of **3** at λ_{max} 263 nm suggested an α,β-unsaturated ketone system. This was further supported by the strong absorption band at 1662 cm^{-1} in the FT-IR spectrum of **3**. The HRFABMS data of **3** showed a molecular ion peak that was less than that of **2** by only 2 mass units and, hence, suggested the molecular formula $C_{27}H_{37}O_2N$. The downfield quaternary carbon resonating at 199.4 ppm was assigned to the α,β-unsaturated ketone group at C-3. The olefinic methine carbon resonating at δ 125.2, which correlated to the proton singlet at δ 5.82, was assigned to C-4 on the basis of the HMBC spectrum. The H-4 signal showed ^3J-correlations with the methylene carbons C-2 and C-6, resonating at 33.8 and 33.2 ppm, respectively, as well as with the quaternary carbon C-10 resonating at 38.4 ppm (Table 1). Proton H-4 showed also a ^2J-correlation with the ketone carbon C-3. The downfield quaternary olefinic carbon resonating at 169.9 ppm was assigned to C-5, which was confirmed by its ^3J−HMBC correlation with the methyl proton singlet C-19 resonating at δ 1.30. In light of the aforementioned spectral data, **3** was assigned as the 4,5-didehydro derivative of compound **2**.

The UV absorption maximum of **4** at λ_{max} 292 and 280 nm suggested an α,β-unsaturated ketone system. The FT-IR spectrum of **4** showed a strong absorption band at 1660 cm^{-1}, which supported this inference. The HRFABMS of **4** exhibited a molecular ion peak that was less than that of **3** by only 2 mass units and, hence, suggested a molecular formula of $C_{27}H_{35}O_2N$. The appearance of an additional double bond was also

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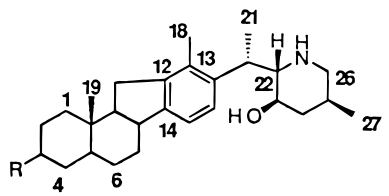
Table 1. ^{13}C- and ^{1}H-NMR Spectral Data of Compounds **2**−**4**[a]

	2		**3**		**4**	
position	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	38.4, t	2.49 m, 2.44 m	36.2, t	2.05 m, 1.52 m	155.5, d	7.02 d (9.9)
2	38.0, t	2.35 m, 1.99 m	33.8, t	2.03 m, 1.91m	127.5, d	6.28 dd (10.0, 1.6)
3	211.4, s		199.4, s		186.8, s	
4	44.0, t	2.21 m, 2.18 m	125.2, d	5.82 s	125.5, d	6.16 dd (1.5, 1.4)
5	46.5, d	1.61 m	169.9, s		167.5, s	
6	28.9, t	2.34 m, 1.51 m	33.2, t	2.56 m, 2.51 m	33.1, t	2.67 m, 2.62 m
7	29.5, t	2.54 m, 1.49 m	29.5, t	2.45 m, 2.40 m	29.8, t	2.76 m, 1.27 m
8	44.1, d	2.89 ddd (11.9, 10.7, 3.6)	43.7, d	3.05 ddd (12.0, 11.6, 3.6)	43.8, d	3.14 ddd (11.8, 11.8, 3.4)
9	60.0, d	1.64 ddd (12.5, 7.0, 4.8)	59.5, d	1.76 m	56.3, d	1.78 ddd (12, 11.8, 6.9)
10	35.6, s		38.4, s		44.0, s	
11	30.5, t	2.75 dd (14.5, 6.8) 1.45 m	30.1, t	2.75 dd (14.5, 6.8) 1.45 m	30.7, t	2.85 dd (14.2, 7.0) 1.44 m
12	142.4, s		142.2, s		140.8, s	
13	132.7, s		132.9, s		132.9, s	
14	143.6, s		142.8, s		141.8, s	
15	119.2, d	6.95 d (7.7)	119.4, d	6.98 d (7.7)	119.3, d	6.96 d (7.8)
16	125.2, d	7.22 d (7.7)	125.5, d	7.24 d (7.7)	125.6, d	7.24 d (7.8)
17	140.3, s		140.6, s		141.5, s	
18	15.8, q	2.30, s	15.8, q	2.31, s	15.7, q	2.31, s
19	11.6, q	1.13, s	16.8, q	1.30, s	18.7, q	1.34, s
20	36.1, d	3.48 dq (7.6, 7.2)	36.1, d	3.49 m	36.0, d	3.48 dq (9.1, 7.0)
21	19.4, q	1.38 d (7.2)	19.5, q	1.38 d (7.2)	18.8, q	1.37d (7.3)
22	67.0, d	2.47 dd (11.9, 7.1)	67.0, d	2.48 m	67.0, d	2.47 dd (9.2, 4.5)
23	70.8, d	3.26 ddd (10.6, 9.2, 4.6)	70.8, d	3.26 ddd (10.4, 9.5 4.6)	70.8, d	3.24 ddd (10.6, 9.3 4.6)
24	44.3, t	2.32m, 0.99 ddd (12.1, 11.1, 4.1)	44.0, t	1.98 m, 1.00 ddd (12.0, 11.2, 4.2)	43.6, t	2.00 m, 1.00 ddd (11.8, 10.9, 4.3)
25	32.6, d	1.44 m	32.1, d	1.44 m	32.1, d	1.43 m
26	54.0, t	2.92 dd (11.9, 3.8) 2.10 dd (11.9, 11.3)	53.9, t	2.92 ddd (12.1, 4.0, 2.0) 2.11 dd (12.0, 11.1)	53.9, t	2.91 ddd (12.0, 4.0, 1.8) 2.11 dd (11.8, 11.3)
27	18.8, q	0.82 d (6.6)	18.8, q	0.82 d (6.6)	19.5, q	0.82 d (6.6)

[a] In $CDCl_3$, at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Carbon multiplicities were determined by DEPT 135 experiments; s = quaternary, d = methine, t = methylene, q = methyl carbons, coupling constants (*J*) are in Hz.

supported by the two new olefinic protons resonating at δ 7.02 d (9.9) and 6.28 dd (10.0, 1.6), which were coupled to each other in the ^{1}H−^{1}H COSY experiment and assigned to H-1 and H-2, respectively. The coupling constants of these protons suggested their *E* orientation. The HMBC spectrum of **4** supported these assignments by showing the following ^{3}J-correlations: H-1 with C-3, C-5, and C-19; H-2 with C-4 and C-10; H-4 with C-10; and H_3-19 with C-1, C-5, and C-9. The HMBC spectrum of **4** showed also ^{2}J-coupling between H-1 and C-2, and H_3-19 and C-10. Consequently, metabolite **4** was assigned the 1,2-didehydro derivative of **3**. It was therefore established that the microbe *Nocardia* species ATCC 21145 was able to metabolize rings A and B of veratramine but failed to metabolize its nitrogen-containing side chain, an observation consistent with previous fermentation studies on steroidal alkaloids.[7,8]



	R	Other
1	β-OH	Δ^{5-6}
2	=O	-
3	=O	Δ^{4-5}
4	=O	Δ^{1-2}; $\Delta^{4,5}$

Of compounds **1**−**4**, only **4** exhibited weak antimalarial activity against *Plasmodium falciparum* W2 clone, with an IC_{50} value of 3600 ng/mL. Compounds **1**−**4** were not cytotoxic to Vero cells.

Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas−Hoover capillary melting point apparatus and are uncorrected. UV spectra were run on a Perkin−Elmer Lambda 3B UV/vis spectrophotometer. The IR spectra were recorded on ATI Mattson Genesis Series FTIR spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$, on a Bruker DRX-400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The HRFABMS spectra were conducted at the University of Kansas on a Fisons/VG Autospec Q mass spectrometer. TLC analysis was carried out on precoated Si gel G_{254} (Merck) or aluminum oxide ALOX-100 UV_{254} (Macherey-Nagel) plates, using the developing system cyclohexanes−EtOAc−diethylamine (2:1:0.01) in both cases. For column chromatography, Baker Si gel 60, 40 μm, was used.

Chemicals. Veratramine (**1**) was purchased from ABC Rare Chemicals, a branch of the Aldrich Chemical Co., Milwaukee, WI.

Organisms. Preliminary microbial metabolism studies were conducted as previously reported.[12] Twenty-five microbial cultures, obtained from the University of Mississippi, Department of Pharmacognosy, culture collection were used for screening. The microbes utilized were *Absidia glauca* ATCC 22752, *Aspergillus flavipes* ATCC 1030, *Aspergillus ochraceus* ATCC 18500, *Aspergillus ochraceus* ATCC 22947, *Aureobasidium pullulans* ATCC 9348, *Beauvaria bassiana* UM-ATCC 7159, *Caldariomyces fumago* ATCC 11925, *Calonectria decora* ATCC 14767, *Chaetomium cochliodes* NRRL 2320, *Cladosporium resinae* ATCC 22712, *Coriolus antarcticus* ATCC 34581, *Cryptococcus neoformans* ATCC 32264, *Cunninghamella blakesleeana* ATCC 8688a,

Cunninghamella elegans ATCC 9245, *Fusarium oxysporium* ATCC 7601, *Fusarium solani* ATCC 12823, *Nocardia* species ATCC 21145, *Penicillium chrysogenum* ATCC 9480, *Rhizopus arrhizus* ATCC 11145, *Rhizopus stolonifer* ATCC 24795, *Saccharomyces cerevisiae* ATCC 2366, *Saccharomyces lipolytica* ATCC 8661, *Streptomyces flocculus* ATCC 25453, *Streptomyces griseus* ATCC 13968, and *Streptomyces lavendulae* L-105.

Microbial Metabolism of Veratramine (1) by *Nocardia* sp. *Nocardia* species ATCC 21145 was grown in two 1-L culture flasks, both containing 250 mL of medium. A total of 68 mg of **1** was dissolved in 0.5 mL EtOH, equally divided between the two flasks and distributed among the 24-h-old stage II cultures. After 14 days, the incubation mixtures were pooled and filtered. The filtrate (0.5 L) was exhaustively extracted with EtOAc (3 × 200 mL), which was then dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue (165 mg) was flash chromatographed over 50 g Si gel 60 starting with (100%) cyclohexane and gradient eluted with increasing proportions of EtOAc and finally with (100%) MeOH. Alkaloid-containing fractions were subjected to repeated preparative TLC on Al_2O_3 to give three metabolites: **2** (4.8 mg, R_f 0.55), **3** (4.7 mg, R_f 0.35), and **4** (3.9 mg, R_f 0.25).

Compound 2: colorless needles from EtOH; mp 168 °C, $[\alpha]^{25}_D$ −12° (*c* 0.1, MeOH); UV λ_{max} (log ϵ) (MeOH) 233 (2.51), 246 (2.43), 266 sh (1.91), 274 (2.03), nm; IR ν_{max} ($CHCl_3$) 3520, 3432 (NH and OH), 3050−2860, 1705 (C=O) cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; LRCIMS *m/z* 410 $[M + H]^+$; HRFABMS *m/z* calcd for $C_{27}H_{40}NO_2$ $[M + H]^+$ 410.3059, found 410.3071.

Compound 3: colorless needles from EtOH; mp 145−146 °C, $[\alpha]^{25}_D$ −2° (*c* 0.1, MeOH); UV λ_{max} (log ϵ) (MeOH) 233 (2.59), 245 (2.48), 263 (2.39),274 (1.94), nm; IR ν_{max} ($CHCl_3$) 3592, 3410 (NH and OH), 3035−2858, 1662 (C=O), 1614 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; LRCIMS *m/z* 408 $[M + H]^+$; HRFABMS *m/z* calcd for $C_{27}H_{38}NO_2$ $[M + H]^+$ 408.2903, found 408.2916.

Compound 4: colorless needles from EtOH; mp 126−127 °C, $[\alpha]^{25}_D$ −41° (*c* 0.1, MeOH); UV λ_{max} (log ϵ) (MeOH) 233 (2.59), 245 (2.49), 280 (2.54), 292 (2.02) nm; IR ν_{max} ($CHCl_3$) 3569, 3463 (NH and OH), 3020−2850, 1672 (C=O), 1622 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; LRCIMS 406 $[M + H]^+$; HRFABMS *m/z* calcd for $C_{27}H_{38}NO_3$ $[M + H_2O + H]^+$ 424.2852, found 424.2852.

Antimalarial Assay. Compounds **1**−**4** were examined for *in vitro* antimalarial activity against the D6 and W2 clones of *Plasmodium falciparum*. The detailed methodology has been described by El Sayed et. al.[13]

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