Microbial Transformation of Rubijervine

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Received March 11, 2002; accepted May 23, 2002

Preparative-scale fermentation of rubijervine (1), the known 22,26-epiminocholestane *Veratrum* alkaloid, with *Cunninghamella echinulata* ATCC 9244 has resulted in the isolation of the new metabolites 7α-hydroxyrubijervine (2) and solanid-5-ene-3β,12α-diol-1-one (3). Structure elucidation of these metabolites was based primarily on 1D- and 2D-NMR analyses. The microbe *C. echinulata* ATCC 9244 was able to metabolize rings A and B of rubijervine but failed to metabolize rings C, D or its *N*-containing side chain, a finding which is analogous to the results of previous fermentation studies of steroidal alkaloids.

Key words biocatalysis; *Veratrum* alkaloid; rubijervine; *Cunninghamella echinulata*; 7α-hydroxyrubijervine; solanid-5-ene-3β,12α-diol-1-one

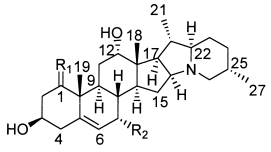
Veratrum alkaloids are a group of potent hypotensive agents that act by reflex suppression of the cardiovascaular system.[2,3)] Rubijervine (**1**) is one of the most common *Veratrum* alkaloids possessing the 22,26-epiminocholestane (solanidane) skeleton.[4)] The interest in *Veratrum* alkaloids has recently been renewed as they were patented several times during the past few years.[5—8)] The use of steroidal alkaloids including solanidanes and C-*nor*-D-homosteroidal alkaloids to reverse or inhibit multidrug resistance in cancer or in bacterial, fungal, or parasite infections was reported.[5)] The potent regulatory effects of jervane *Veratrum* alkaloids on hedgehog signaling, modulation of cholesterol biosynthesis and transport, and control of cell proliferation during mandibular arch morphogenesis have recently been reported.[6—8)] Rubijervine and isorubijervine are suggested to be the plausible biogenetic precursors of cevanine and other C-*nor*-D-homosteroids.[9)] Rubijervine is classified as a possible carcinogen for humans, based on its high tg α value when evaluated by the DC polarography method in anhydrous *N*,*N*-dimethylformamide.[10)] The antimicrobial activity of rubijervine and other *Veratrum* alkaloids against *Pityrosporum ovale*, *Trichophyton mentagrophytes*, and *Saccharomyces cerevisiae* and against the basidiomycetes *Polstictus versicolor* has been reported.[11,12)]

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.[13)] Steroidal alkaloids have been investigated in metabolism studies due to their interesting biological activities.[14)] Tomatidine is a known spirosolane-type *Solanum* alkaloid related to rubijervine.[15)] Incubation of tomatidine 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.[15)] Solanidine (12-deoxyrubijervine) was the subject of a previous microbial metabolism study which enabled its conversion into 11α-hydroxysolanidine using the fungus *Helicostylum piriforme* ATCC 8992.[16)] The microbes *Nocardia* species ATCC 21145 and *Cunninghamella elegans* ATCC 9245 were able to metabolize rings A and B of the C-*nor*-D-homosteroidal *Veratrum* alkaloids veratramine and jervine, respectively, but failed to metabolize rings C and D or its *N*-containing side chain.[17,18)] Rubijervine (**1**) was chosen for a microbial bioconversion study in an attempt to prepare new analogues, to determine if there was any metabolism in the *N*-containing side chain, and to compare its metabolism with that of solanidine, tomatidine, veratramine, and jervine.

Twenty-three growing cultures were screened for their ability to bioconvert **1**. Only one culture (*C. echinulata* ATCC 9244) was observed to transform **1** completely to metabolites of greater polarity. Hence this microbe was chosen for preparative-scale fermentation because it entirely depleted **1** and converted it to the more polar metabolites **2** and **3**.

The high-resolution FT-ICR MS of **2** displayed a peak at *m*/*z* 430.3341 $(M+H)^+$, suggesting the molecular formula $C_{27}H_{43}O_3N$, and seven degrees of unsaturation. The IR, ^{13}C-, and ^1H-NMR spectra of **2** (Table 1) suggested that it is a monohydroxylated rubijervine derivative.[4,19)] The broad proton singlet resonating at δ 3.57 is assigned to be the newly oxygenated H-7. This was based on its COSY coupling with the olefinic H-6 (δ_H 5.35) and H-8 (δ_H 1.42). The proton H-6 also displayed a ^2J-HMBC coupling with C-7 (δ_C 64.3). The splitting pattern of H-7 indicated its equatorial nature, suggesting a β-orientation. This was further confirmed by the NOESY correlation between H-7 and the β-oriented H-8. Hence metabolite **2** is 7α-hydroxyrubijervine, a new natural product.

The high-resolution FT-ICR MS of **3** displayed a peak at *m*/*z* 428.3169 $(M+H)^+$, suggesting the molecular formula $C_{27}H_{41}O_3N$, and eight degrees of unsaturation. The IR, ^{13}C-, and ^1H-NMR spectra of **3** (Table 1) suggested it is a rubijervin-1-one derivative. The strong IR band at 1710 cm^{-1} in-



	R_1	R_2
Rubijervine (**1**)	H_2	H
7α-Hydroxyrubijervine (**2**)	H_2	OH
Solanid-5-ene-3β,12α-diol-1-one (**3**)	O	H

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Table 1. ^{13}C- and ^{1}H-NMR Spectral Data of 7α-Hydroxyrubijervine (**2**) and Solanid-5-ene-3β,12α-diol-1-one (**3**)[a)]

Position	**2**		**3**	
	δ_C	δ_H	δ_C	δ_H
1	36.8	1.55, m 1.49, m	213.6	—
2	32.2	1.62, m 1.30, m	37.9	2.43, m 2.01, m
3	70.7	3.24, m	71.2	3.20, m
4	43.1	2.12, m 2.09, m	43.9	2.14, m 2.06, m
5	143.5	—	140.3	—
6	125.6	5.35, d (3.4)	126.2	5.21, d (5.2)
7	64.3	3.59, br s	32.6	1.64, m 1.31, m
8	38.3	1.42, m	32.0	1.48, m
9	40.7	2.07, m	50.5	1.63, m
10	37.4	—	37.0	—
11	29.0	1.47, 2H, m	34.5	1.62, m 0.79, m
12	71.2	3.47, br s	71.9	3.62, br s
13	44.6	—	44.0	—
14	41.8	2.08, m	42.8	2.11, m
15	31.5	1.85, m 0.94, m	30.5	1.65, m 1.11, m
16	69.1	2.52, ddd (10.4, 10.1, 3.2)	69.1	2.51, m
17	53.9	2.20, dd (10.4, 10.3)	55.0	2.18, m
18	18.1	0.70, 3H, s	20.5	0.73, 3H, s
19	18.7	0.84, 3H, s	19.7	1.01, 3H, s
20	37.1	1.62, m	38.1	1.58, m
21	19.0	0.88, 3H, d (6.1)	19.1	0.93, 3H, d (5.9)
22	75.1	1.48, m	75.2	1.46, m
23	29.6	1.64, m 1.20, m	29.7	1.49, m 1.20, m
24	33.9	1.62, m 0.78, m	34.5	1.62, m 0.79, m
25	31.6	1.47, m	31.6	1.47, m
26	60.5	2.81, dd (11.0, 3.5) 1.31, dd (11.0, 3.4)	60.9	2.77, dd (10.8, 3.4) 1.32, m
27	20.3	0.78, 3H, d (6.6)	20.1	0.78, 3H, d (6.3)

a) In DMSO-d_6, at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Coupling constants (*J*) are in Hz.

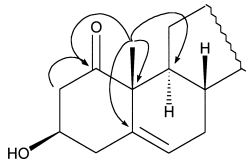

Fig. 1. Important HMBC Correlations in Rings A and B of Metabolite **3**

dicated a new ketone functionality. The quaternary ketone carbon resonating at δ 213.6 was assigned to be C-1 (Table 1). This was based on its ^{3}J-HMBC coupling with the methyl singlet H_3-19 (δ_H 1.01) and ^{2}J-HMBC coupling with both protons H_2-2 at δ 2.43 and 2.01 (Fig. 1). The methyl H_3-19 also shows ^{3}J-HMBC coupling with C-5 and C-9 as well as a ^{2}J-HMBC coupling with the quaternary carbon C-10 (Fig. 1). Metabolite **3** is thus confirmed to be the new solanid-5-ene-3β,12α-diol-1-one, a new metabolic product reported for the first time in fermentation studies of steroidal alkaloids. Hence the microbe *C. echinulata* ATCC 9244 was able to metabolize rings A and B of rubijervine but failed to metabolize rings C and D or its *N*-containing side chain, a finding which is analogous to the results of previous fermentation studies of steroidal alkaloids.

Experimental

General Experimental Procedure Melting points were determined on a Thomas–Hoover capillary melting point apparatus and are uncorrected. The IR spectra were recorded on a ATI Mattson Genesis Series FTIR spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded in DMSO-d_6 on a Bruker AMX-500 spectrometer operating at 500 MHz for ^{1}H- and at 125 MHz for ^{13}C-NMR. The HRMS spectra were measured on a Bioapex FT-ICR MS with electrospray ionization. TLC analyses were carried out on precoated silica gel G_{254} 500 μm (E-Merk), with the following developing system: $CHCl_3$–MeOH–NH_4OH (80 : 20 : 0.01). For column chromatography, Si gel 60 and 40 μm was used.

Chemicals Rubijervine (**1**) was isolated from the roots and rhizomes of *Veratrum viride* Aiton.[4)] The compound was authenticated by detailed NMR analysis and comparing its data with those in the literature.[19)]

Organisms Preliminary microbial metabolism studies were conducted as previously reported.[17,18,20)] Twenty-three microbial cultures, obtained from the Department of Pharmacognosy, University of Mississippi, culture collection were used for screening. The microbes utilized were reported earlier,[20)] in addition to: *Cunninghamella* species NRRL 5695 and *C. echinulata* ATCC 9244. Stock cultures were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C.

Microbial Metabolism of Rubijervine (1) *C. echinulata* ATCC 9244 was grown in three, 1-l culture flasks containing 200 ml of compound medium α.[18)] A total of 50 mg of **1** was dissolved in EtOH 1 ml, equally divided between the three flasks and distributed among the 24-h old stage II cultures. After 14 d, the incubation mixtures were pooled and filtered. The filtrate (0.5 l) was exhaustively extracted with 10% MeOH/$CHCl_3$ (3× 300 ml), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue (290 mg) was flash-chromatographed over 50 g of silica gel 60 starting with (100%) *n*-hexanes and gradient-eluted with increasing proportions of acetone and finally with (100%) MeOH. Alkaloid-containing fractions (48 mg) were subjected to column chromatography over Sephadex LH20 50 g using an isocratic CH_2Cl_2–MeOH (50 : 50) solvent system. Semi-pure compounds were finally purified by preparative TLC on Si gel G_{254} using $CHCl_3$–MeOH (75 : 25) as a solvent system to give two metabolites: **2** (4.9 mg, *Rf* 0.33) and **3** (2.9 mg, *Rf* 0.59).

7α-Hydroxyrubijervine (**2**): Colorless needles from EtOH, mp 231—232 °C, $[\alpha]_D^{25}$ +3.5° (c=0.1, MeOH); IR ν_{max} ($CHCl_3$): 3547 (NH), 3027—2860, 1450, 1342, 1055 cm^{-1}; ^{13}C- and ^{1}H-NMR, see Table 1; LR-EIMS *m/z* 429 $(M)^+$; FT-ICR MS *m/z* Calcd for $C_{27}H_{44}O_3N$ $(M+H)^+$ 430.3321; Found 430.3341.

Solanid-5-ene-3β,12α-diol-1-one (**3**): Colorless needles from MeOH, mp 241—243 °C, $[\alpha]_D^{25}$ +35° (c=0.1, MeOH); IR ν_{max} ($CHCl_3$) 3698 (OH),

3596 (NH), 3021—2856, 1710 (C=O), 1458, 1361, 1060 cm^{-1}; ^{13}C- and ^{1}H-NMR, see Table 1; LR-EIMS *m/z* 427 $(M)^+$; FT-ICR MS *m/z* Calcd for $C_{27}H_{42}O_3N$ $(M+H)^+$ 428.3165; Found 428.3169.

Acknowledgment This paper is dedicated to Professor Ahmed F. Halim, Mansoura University, on his 60th birthday.

References and Notes

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