Salvinorins J from *Salvia divinorum*: Mutarotation in the Neoclerodane System

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A search for biosynthetic precursors of salvinorin A (**1**) led to the isolation of a new neoclerodane diterpenoid hemiacetal mixture, salvinorins J (**2**), from the chloroform extract of *Salvia divinorum.* A leaf surface extraction method was used on *S. divinorum*, affording a chlorophyll-free extract containing predominantly neoclerodane diterpenoids, including the new salvinorins J (**2**) and 14 known analogues. Salvinorins J (**2**) represent an example of a neoclerodane hemiacetal (lactol) susceptible to mutarotation with the formation of an equilibrium mixture of C-17 epimers.

Salvia divinorum Epling & Jativa-M. (Lamiaceae) has captured the interest of researchers and the general public because of its profound psychopharmacological properties. The wide use of this plant by adolescents[1–3] has raised concerns over its safety and pharmacological side effects, leading to a ban on the possession and selling of the plant in several U.S. states and in various countries worldwide.[4] Native to the Oaxaca region of Mexico, the ethnobotanical use of *S. divinorum* has been practiced for centuries.[5] The psychoactive component of the plant, the neoclerodane diterpenoid salvinorin A (**1**), has been studied intensively as a potent and selective κ-opioid agonist.[6] Several natural analogues of **1** were isolated and structurally described,[7–12] and the biosynthesis of salvinorin A has recently been elucidated as proceeding through the deoxyxylulose phosphate (DOXP) pathway.[13]

In our continuing study of the biosynthesis of salvinorin A (**1**) we have developed an efficient and rapid method of extraction of *S. divinorum* metabolites by simple dipping of the fresh leaves in $CHCl_3$. This method of extraction has provided predominantly secondary metabolites with salvinorin- and divinatorin-like structures and is based on the fact that **1** and its natural analogues are located in the glandular trichomes on the leaf surface.[14] Thus, we speculated that the biosynthesis of the diterpenoids may occur at the same location and that exhaustive extraction of dry plant material may not be a prerequisite for a salvinorin-rich extract. The crude extract obtained by leaf-surface extraction afforded 2.4 g of pure **1**, i.e., 0.024% of fresh weight or 0.2% of dry weight (see Experimental Section). This yield is comparable with those from the "conventional" extraction method of using dried plant material (0.1−0.3%).[14] One of the advantages of the leaf-surface extraction method over the traditional methods[11,12,15,16] is that the extract is virtually chlorophyll-free. Comparing to other methods of decoloration, such as passage over activated charcoal or Celite, the dipping method simplifies the process of purification, saving time and resources. The compounds isolated and their yields are collated in Table S1, Supporting Information. The surface extraction method was previously reported for other plants as a superior method of isolation of furanocoumarins from *Ruta graveolens*[17] or flavonols from *Nicotiana attenuatae*.[18]

After separation of salvinorin A (**1**) from the extract by crystallization from EtOH, the mother liquor was defatted by partitioning between MeCN and hexanes to yield an MeCN and a hexane fraction. The MeCN fraction was redissolved in MeOH and resolved on a silica gel column into 15 groups of fractions (see Experimental Section and Supporting Information). These groups were separately injected onto reversed-phase HPLC to afford salvinorins J (**2**) (17α- and 17β-isomers)[19] and 14 known analogues (see Table S1, Supporting Information).

Salvinorins J (**2**) were obtained as a colorless solid, $[\alpha]^{23.7}_D$ 16.5. This mixture gave a sodiated molecular ion peak at *m*/*z* 457.1839 $[M + Na]^+$ that corresponded to a molecular formula of $C_{23}H_{30}O_8Na$ (calcd 457.1833). The ^{1}H NMR spectrum showed the typical pattern of resonances for salvinorins, in particular the three furan protons at δ 7.44 (H-16), 7.40 (H-15), and 6.44 (H-13) and the methyl groups H_3-23 at δ 3.75, H_3-19 at δ 1.68, and H_3-20 at δ 1.21. As in all salvinorin and divinatorin derivatives bearing a hydroxy group at C-1, compound **2** also showed a distorted doublet of doublets at δ 4.42. The NOESY spectrum revealed coupling between H-1_{eq}, H-2_{ax}, and H-10_{ax}, indicating an α-orientation of the hydroxy group. A three-proton resonance showing an HMBC correlation with C-2 at δ 2.14 suggested the presence of a C-2 *O*-acetyl group. Thus, salvinorins J closely resemble their apparent biosynthesis precursors, salvinorins I.[10] Analysis of the ^{1}H NMR spectrum of **2** consistently showed admixture with ca. 30% of the α-epimer, on the basis of the integration of nonoverlapping resonances of H-17 at δ 5.15 and H-12 at δ 5.23. Attempts to separate this mixture invariably failed, suggesting the presence of an equilibrium mixture in solution (ca. 10% of the α-epimer, based on the HPLC chromatogram). Such a discrepancy between the ratio of β- and α-epimers is explicable in terms of an equilibrium shift in the different solvent systems used. Indeed, inspection of the ^{1}H and ^{13}C NMR spectra showed a near equivalent set of resonances albeit with lower intensity (see Supporting Information). Major differences in both chemical shifts and coupling constants were visible in the H-12 and H-17 regions. This suggests epimerization at C-17 occurred during chromatographic manipulation, as evidenced by the emergence of a weakly resolved doublet of doublets at δ 5.23 and a broad singlet at δ 5.12. The six-membered acetal-type moiety of **2** facilitates epimerization at C-17 in a process similar to mutarotation in, for example, the glucopyranosides,[20] to produce a mixture of C-17 epimers in which the isomer with the β-oriented equatorial hydroxy group predominates as thermodynamically more stable (Scheme 1).

Biosynthetically, salvinorins J (**2**) are most probably derived from salvinorins I by the action of an acetyltransferase (Scheme S1, Supporting Information). Although salvinorins I have been reported before,[10] the authors did not mention their susceptibility to epimerization at C-17. However, our sample of salvinorins I isolated from *S. divinorum* displayed the same NMR characteristics of a C-17 hemiacetalic mixture of C-17 epimers as salvinorins J. Shirota et al. postulated that salvinorins I are derived biosynthetically from salvinorin H through reduction of the C-17 carbonyl group to a lactol functionality.[16] On the contrary, we are postulating that salvinorins I may feasibly be derived from divinatorin E via an oxygenation/acetalization mechanism. Thus, cytochrome P450

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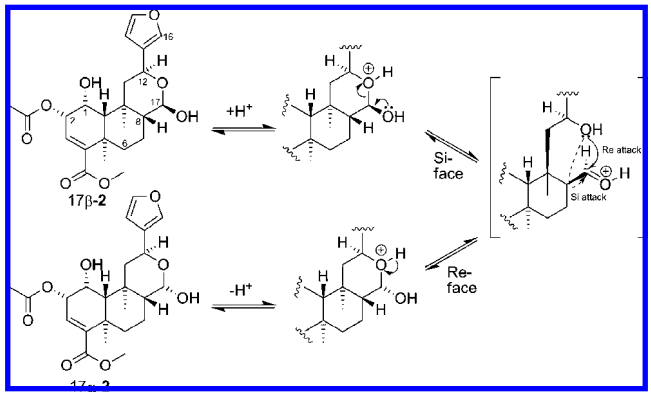
Scheme 1. Mechanism of Acid-Catalyzed Epimerization at C-17 in Salvinorins J (**2**)



oxygenation of divinatorin E at C-12 may afford the intermediate C-12 secondary alcohol that will be susceptible to facile acetalization (lactolization) into the lactol. Subsequent oxygenation at C-2 and dehydrogenation of the C-17 lactol functionality will then result in the formation of the lactone moiety of salvinorin H (Scheme S1, Supporting Information).

Thus, we have demonstrated the presence of a C-17 epimeric mixture of the new hemiacetal salvinorins J, an inherently inseparable mixture evidenced by the duplication of certain ^{1}H and ^{13}C NMR resonances. Salvinorins J may feasibly represent key intermediates in a novel biosynthesis pathway to salvinorin A (**1**) via salvinorins I.

Experimental Section

General Experimental Procedures. The optical rotation was measured using an Autopol IV digital polarimeter (Rudolph Scientific Analitical). The UV spectrum was obtained on a Shimadzu model UV3101PC spectrophotometer, and the IR spectrum recorded using a Bruker Tensor 27 spectrometer. 1D NMR spectra were obtained using a Bruker AV 400 spectrometer equipped with a tunable carbon sensitive 3 mm probe, whereas 2D NMR spectra were obtained using a Bruker DRX 500 spectrometer equipped with a 1 mm microprobe. All NMR spectra were calibrated to deuterium in $CDCl_3$. Mass spectra of pure compounds were analyzed by HRTOFMS (Bruker Daltonik microTOF, Leipzig, Germany). All chemicals for this study were purchased from Fisher Scientific (Pittsburgh, PA). Silica gel flash grade 40−60 μm (230 × 400 mesh) was purchased from Sorbent Technologies (Atlanta, GA). TLC was performed with Merck HF_{254} silica gel plates, developed using a mobile phase composed of 1:1 hexanes and EtOAc, and visualized by dipping in a 10% solution of vanillin in EtOH followed by heating at 120 °C.

Plant Material. *Salvia divinorum* plants were purchased in August 2008 from Salvia divinorum Research and Information Center and identified by Daniel Siebert (Malibu, CA). The plants were cultivated in a greenhouse (in Oxford, MS) in one-gallon pots under normal growth conditions and were weekly fertilized with 10 g of Osmocote (Scott-Sierra, Horticulture Products Co., Marysville, OH). Plants were harvested, immediately placed in bags, and stored at 4 °C until extraction with $CHCl_3$. A voucher specimen, number FH4-408-08, was deposited in the Department of Pharmacognosy plant repository.

Extraction and Isolation. About 10 kg of the fresh aerial parts of the plant was collected and used for extraction. Freshly cut material (10−30 cm long) was individually dipped into a 1000 mL beaker filled with $CHCl_3$ for 1 min with gentle stirring. After about 0.5 kg of plant material was used, a fresh portion of $CHCl_3$ was used and the procedure continued until the entire stock of plant material was extracted. Evaporation of the solvent in vacuo gave a yellowish resin-like material (8.54 g). The dried extract was dissolved in EtOH to crystallize salvinorin A (2.0 g). The mother liquor was dissolved in MeCN and partitioned with hexanes to give 0.22 g of a hexane fraction and 2.46 g of an MeCN fraction. The MeCN fraction was dissolved in MeOH to give an additional 0.31 g of **1** and 2.17 g of a MeOH-soluble fraction. The MeOH fraction was then subjected to open column chromatography and loaded onto 200 g of silica gel (700 × 50 mm, flow rate 10 mL/min) and chromatographed with acetone and CH_2Cl_2 as mobile phase in a gradient mode (2−40% of acetone), followed by MeOH. Approximately 460 fractions were collected, which were subsequently combined based on their TLC profiles (10% acetone in CH_2Cl_2) to give 15 final fractions (Sd-1−Sd-15). Individual fractions were injected onto analytical HPLC equipped with a Luna C18(2) 250 × 4.6 mm, 5 μm particle size column (Phenomenex, Torrance, CA), and MeCN (A) and H_2O (B) mobile phase in gradient mode: 20% of A to 100% of A over 240 min. The injection volume was 10 μL (10 mg/mL), and a flow rate of 1 mL/min at ambient temperature (Supporting Information) was used. Purification of the compounds listed in Table S1 Supporting Information was performed using preparative HPLC under the same chromatographic conditions as used in the analytical mode with the exception that a Luna C18(2) 250 × 15 mm, 5 μm particle size column was used, and the flow rate was 10 mL/min.

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts of Salvinorins J

position	δ_C, mult.	δ_H (J in Hz)
1	69.2, CH [69.3 CH][a]	4.42 br s [4.41 br s][a]
2	67.1, CH [66.5 CH]	5.66 d (5.2) [5.6 s]
3	135.3, CH	6.52 d (4.5) [6.51 d (4.5)]
4	141.9, qC	
5	38.0, qC	
6	38.2, CH2	2.50 d (13.1) 1.25 m
7	17.6, CH2	1.39 m 1.87 dd, (13.9, 2.8) [1.95 dd (13.9, 2.8)]
8	50.12, CH	1.47 d (12.3 Hz)
9	36.8, qC	
10	53.7, CH	1.34 s
11	45.6, CH2 [46.0 CH2]	1.99 dd (12.8, 2.3) [2.04 dd (12.8, 2.3)] 1.29 s [1.33 s]
12	66.3, CH [61.4 CH]	4.91 dd (11.5, 1.9) [5.23 d (9.9)]
13	126.4, qC	
14	108.8, CH [108.8, CH]	6.44 s [6.42 s]
15	143.2, CH [141,9 CH]	7.40s [7.38 s]
16	139.2, CH	7.44 s
17	94.4, CH [96.0 CH]	4.80 dd (6.30, 8.71) [5.15 t (2.7)]
18	166.5, qC	
19	22.2, CH3	1.68 s
20	15.8, CH3	1.21 s
*C*OCH$_3$	171.7, qC	
CO*CH$_3$*	21.3, CH3	2.14 s
COO*CH$_3$*	51.6, CH3 [50.4 CH3]	3.75 s [3.76 s]

[a] Whenever possible, chemical shifts were assigned for the C-17 epimer with an α-oriented hydroxy group.

Salvinorins J: colorless solid; $[\alpha]^{23.8}_D$ 16.5 (c 0.23 $CHCl_3$); UV (MeCN) λ_{max} (log ε) 211 (4.08) nm; FTIR (film) ν_{max} 3407, 2950, 1717, 1232, 1026 cm^{-1}; ^{1}H (400 and 500 MHz, $CDCl_3$) and ^{13}C NMR (100 and 125 MHz, $CDCl_3$) data, Table 1; HRTOFMS m/z [M + Na]$^+$ 457.1839 (calcd for $C_{23}H_{30}O_8Na$ 457.1833).

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Supporting Information Available: HPLC chromatograms of groups of fractions, 1D and 2D NMR spectra, HRMS, UV, and IR spectrum of salvinorins J, table with isolated metabolites from dipping extraction method, and the proposed biosynthesis scheme leading to the formation of salvinorins J (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Boyer, E. W.; Shannon, M.; Hibberd, P. L. *Pediatrics* **2005**, *115*, 302–305.
(2) Dennehy, C. E.; Tsourounis, C.; Miller, A. E. *Ann. Pharmacother.* **2005**, *39*, 1634–1639.
(3) Babu, K. M.; McCurdy, C. R.; Boyer, E. W. *Clin. Toxicol.* **2008**, *46*, 146–152.
(4) Griffin, O. H.; Miller, B.; Lee; Khey, D. N. *J. Psychoact. Drugs* **2008**, *40*, 183–91.
(5) Valdes, L. J.; Diaz, J. L.; Paul, A. G. *J. Ethnopharmacol.* **1983**, *7*, 287–312.

(6) Roth, B. L.; Baner, K.; Westkaemper, R.; Siebert, D.; Rice, K. C.; Steinberg, S.; Ernsberger, P.; Rothman, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11934–1193.
(7) Valdes, L. J., III.; Butler, W. M.; Hatfield, G. M.; Paul, A. G.; Koreeda, M. *J. Org. Chem.* **1984**, *49*, 4716–20.
(8) Valdes, L. J., III.; Chang, H.-M.; Visger, D. C.; Koreeda, M. *Org. Lett.* **2001**, *3*, 3935–3937.
(9) Lee, D. Y. W.; Ma, Z.; Liu-Chen, L.-Y.; Wang, Y.; Chen, Y.; Carlezon, W. A.; Cohen, B. *Bioorg. Med. Chem.* **2005**, *13*, 5635–5639.
(10) Shirota, O.; Nagamatsu, K.; Sekita, S. *J. Nat. Prod.* **2006**, *69*, 1782–1786.
(11) Bigham, A. K.; Munro, T. A.; Rizzacasa, M. A.; Robins-Browne, R. M. *J. Nat. Prod.* **2003**, *66*, 1242–1244.
(12) Munro, T. A.; Rizzacasa, M. A. *J. Nat. Prod.* **2003**, *66*, 703–705.
(13) Kutrzeba, L.; Dayan, F. E.; Howell, J. L.; Feng, J.; Giner, J.-L.; Zjawiony, J. K. *Phytochemistry* **2007**, *68*, 1872–1881.
(14) Siebert, D. *Ann. Bot.* **2004**, *93*, 763–771.
(15) Tidgewell, K.; Harding, W. W.; Schmidt, M.; Holden, K. G.; Murry, D. J.; Prisinzano, T. E. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5099–5102.
(16) Shirota, O.; Nagamatsu, K.; Sekita, S. *J. Liq. Chromatogr. Relat. Technol.* **2007**, *30*, 1105–1114.
(17) Zobel, A. M.; Brown, S. A. *J. Nat. Prod.* **1988**, *51*, 941–6.
(18) Roda, A. L.; Oldham, N. J.; Svatos, A.; Baldwin, I. T. *Phytochemistry (Elsevier)* **2003**, *62*, 527–536.
(19) We use the plural, salvinorins J, to describe an inherently inseparable hemiacetalic mixture of C-17 epimers. For the same reason, salvinorin I should also be called salvinorins I.[16]
(20) Jouppila, K. *Food Sci. Technol. (N. Y.)* **2006**, *159*, 41–88.

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