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Bioorganic & Medicinal Chemistry 13 (2005) 5635–5639
Bioorganic & Medicinal Chemistry

New neoclerodane diterpenoids isolated from the leaves of *Salvia divinorum* and their binding affinities for human κ opioid receptors

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Received 14 March 2005; revised 11 May 2005; accepted 18 May 2005
Available online 9 August 2005

Abstract—Bioactivity-guided fractionation of the leaves of *Salvia divinorum* has resulted in the isolation of three new neoclerodane diterpenoids: divinatorin D (**1**), divinatorin E (**2**), and salvinorin G (**3**), together with 10 known terpenoids, divinatorin C (**4**), hardwickiic acid (**5**), salvinorin-A (**6**), -B (**7**), -C (**8**), -D (**9**), -E (**10**), and -F (**11**), presqualene alcohol (**12**), and (*E*)-phytol (**13**). The structures of these three new compounds were characterized by spectroscopic methods. All these compounds were evaluated for their binding affinities to the human κ opioid receptors. In comparison with divinatorin D (**1**), divinatorin E (**2**), and salvinorin G (**3**), salvinorin A (**6**) is still the most potent κ agonist.
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1. Introduction

The kappa (κ) opioid receptor (KOR) is one of three main types of opioid receptors. It is differentiated from mu (μ) and delta (δ) opioid receptors by distinct genes and proteins, tissue expression patterns, functional properties, and pharmacological profiles of selective agonists and antagonists.[1] Activation of the KOR in vivo produces numerous effects, including analgesia, dysphoria, corticosteroid elevation, diuresis, antipruritic effect, immunomodulation, and decreases in pilocarpine-induced seizures and associated mossy fiber sprouting and hilar neuron loss.[2] The KOR also participates in the expression of chronic morphine-induced withdrawal syndrome and mediates the aversive effects of Δ9-tetrahydrocannabinol.[3,4] The synthetic arylacetamides, U50,488H, U69,593, spiradoline, enadoline, ICI 204448, and asimadoline, are selective KOR agonists.[5,6] Interestingly, the KOR agonist U69,593 produces depressive-like effects, and the KOR antagonists nor-BNI and ANTI produce antidepressant-like effects in animal models.[7,8] Furthermore, KOR agonists appear to affect mood in humans.[9,10] In addition, several other compounds, such as TRK 820 and HZ2, were reported to be KOR agonists and may be useful as analgesics, water diuretics, and antipruritics.[11–14] Recently, salvinorin A was identified as a KOR agonist.[15–19]

As part of our ongoing investigation, we initiated a comprehensive fractionation study guided by KOR binding activity in an attempt to find new KOR agonists or antagonists from *Salvia divinorum* (Lamiaceae). *S. divinorum* is a traditional medicine of the Mazatec Indians of Oaxaca, Mexico.[20] It has been used for many hundreds of years, primarily for its psychoactive (hallucinogenic) effects in their divination rites. An extract prepared from the crushed leaves induces 'visions' and the psychotropic effects, including effects on mood, have been verified by a number of researchers.[21] Salvinorin A, a nonnitrogenous neoclerodane diterpenoid, was isolated and identified to be the key ingredient for these

Keywords: κ Opioid receptor; *Salvia divinorum*; Salvinorin A; Diterpenoid.
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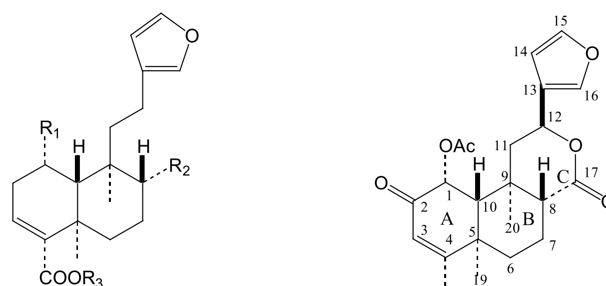
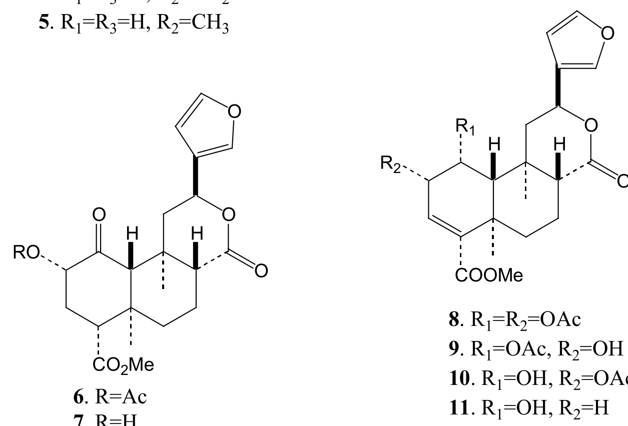
doi:10.1016/j.bmc.2005.05.054

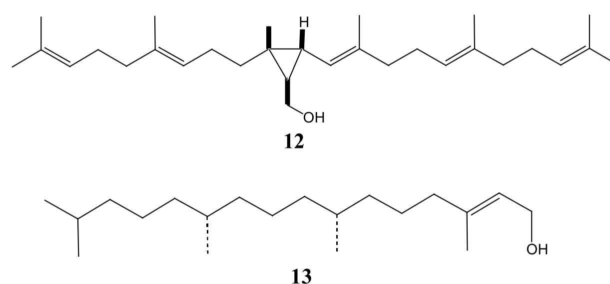
psychoactive effects.[22] In addition, salvinorin A was confirmed to have very high affinity and selectivity for the KOR at nanomolar concentrations.[15–19] Previous phytochemical investigations of this plant have resulted in the isolation of 15 terpenoids, including salvinorins A–F and divinatorins A–C.[21,23–26] Herein, we report the isolation and characterization of three new diterpenoids (**1**, **2**, and **3**) and 10 known terpenoids (**4**–**13**), and their KOR binding activities.



1. R_1=OH, R_2=CH_2OAc, R_3=CH_3
2. R_1=OH, R_2=CHO, R_3=CH_3
4. R_1=R_3=H, R_2=CH_2OAc
5. R_1=R_3=H, R_2=CH_3

3





2. Results and discussion

The dried leaves (4.6 kg) of *S. divinorum* were sequentially extracted with hexane, acetone, and methanol. The acetone extract showed the most potent binding activity to human KOR; the hexane and methanol fractions were much less potent. Following published procedures,[23,24] the pigments in acetone extract were removed by chromatography on activated carbon. Upon further recrystallization from acetone and methanol, a significant amount of salvinorin A (~10 g) was isolated. The supernatant of the acetone extract was chromatographed on a silica gel column and eluted with chloroform–acetone to give five fractions. The fraction eluted with chloroform–acetone (20:1) had the most potent binding activity and was subjected to repeated silica gel column chromatography to give three new diterpenoids: divinatorin D (**1**), divinatorin E (**2**), and salvinorin G (**3**), and 10 known compounds, divinatorin C (**4**), hardwickiic acid (**5**), salvinorin-A (**6**), -B (**7**), -C (**8**), -D (**9**), -E (**10**), and -F (**11**), presqualene alcohol (**12**), and (*E*)-phytol (**13**). The known compounds were identified by comparison with their published data.[21,23–26]

Compound **1** was isolated as a colorless semi-solid with $[\alpha]_D^{23}$ −172° (*c* 0.10, $CHCl_3$). Its molecular formula was determined to be $C_{23}H_{32}O_6$ on the basis of the ion peak at *m*/*z* 404.2187 $[M]^+$ in the HR EI-MS spectrum. The ^1H and ^{13}C NMR data of **1** were closely related to those of divinatorin B,[24] except for additional signals arising from the acetyl group. The acetyl group was placed at C-17 by the observation of ester linkage which causes a downfield shift of H-17 in the ^1H NMR spectrum as compared with that of divinatorin B. This was further evidenced by ^{13}C–^1H long-range correlation in the HMBC spectrum (Fig. 1), in which the correlations were clearly established between H-17 (δ 3.80 and 4.28) and C-7 (δ 22.3), C-8 (δ 41.4), C-9 (δ 39.1), and carbonyl carbon (δ 171.3) in the acetyl moiety. The ^1H and ^{13}C signals of **1** were assigned by extensive 2D-NMR methods (Table 1). Consequently, the structure of **1** was proposed for divinatorin D.

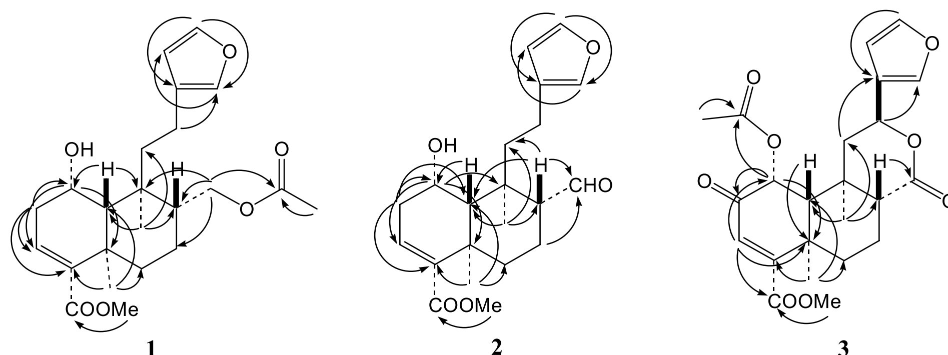


Figure 1. Selected HMBC correlations of **1**, **2**, and **3**.

Table 1. NMR data for **1**, **2**, and **3** in $CDCl_3$[a,b]

Carbon No.	**1**		**2**		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	64.2	4.48 d (4.8)	63.8	4.48 d (4.5)	68.5	5.80 d (3.0)
2	38.0	2.55 ddd, (19.5, 5.1, 2.4) 2.32 m	38.0	2.58 ddd, (19.8, 5.1, 2.7) 2.34 m	192.9	
3	133.3	6.76 dd (4.5, 3.0)	133.5	6.71 dd (3.9, 3.0)	127.9	6.41 br s
4	141.3		140.9		161.2	
5	37.1		37.0		38.2	
6	37.8	2.38 m 1.13 dd(13.5, 3 6)	36.8	2.49 m 1.15 dd (13.2, 4.5)	35.3	2.32 dt (13.5, 3.3) 1.36 dd (13.2, 3.3)
7	22.3	1.73 m 1.58 dd (13.5, 3.6)	19.0	2.06 m 1.67 m	18.2	2.20 m 1.82 dd (13.5, 3.3)
8	41.4	1.70 m	55.1	2.33 ddd (13.2, 3.9, 3.6)	52.6	2.18m
9	39.1		39.7		36.8	
10	48.7	1.45 s	48.6	1.44 s	53.8	1.91 d(3.0)
11	39.1	2.07 dt (13.8, 4.5) 1.76 m	40.8	2.02 dt (12.6, 4.2) 1.76 dt (12.3, 3.6)	43.2	2.58 dd (13.2, 3.3) 1.74 br d (12.6)
12	18.2	2.42 dt (13.8, 4.5) 1.93 dt(12.9, 4.5)	18.4	2.49 m 2.12 m	71.8	5.51 dd (11.7, 5.4)
13	124.8		124.4		125.1	
14	110.8	6.28 br s	110.8	6.28 br s	108.3	6.41 br s
15	142.9	7.35 br s	143.0	7.36 t(1.5)	143.9	7.42 br s
16	138.5	7.22 br s	138.6	7.22 br s	139.5	7.44 br s
17	65.9	4.28 dd (10.8, 3.9) 3.80 dd (10.8, 8.4)	205.8	9.74 d (3.6)	169.7	
18	167.2		167.0		166.2	
19	21.4	1.66 s	21.3	1.66 s	22.8	1.78 s
20	20.7	1.23 s	21.4	1.43 s	16.0	1.27 s
COCH_3	171.3				170.9	
COCH_3	21.0	2.02 s			21.3	2.14 s
COOCH_3	51.3	3.71s	51.4	3.72 s	52.1	3.84 s

[a] Chemical shifts in ppm, *J* values in Hz are in parentheses.
[b] Assignments were made using ^{1}H–^{1}H COSY, HMQC, and HMBC techniques.

Compound **2** was obtained as a colorless semi-solid. The molecular formula of $C_{21}H_{28}O_5$ was established by HR EI-MS. The ^{1}H NMR spectrum revealed the presence of two tertiary methyl groups (δ 1.43 and 1.66), a methoxyl group (δ 3.72), an oxygenated methine proton (δ 4.48), four olefinic protons (δ 6.28, 6.71, 7.22, and 7.36), and an aldehyde proton (δ 9.74). The ^{13}C NMR spectrum of **2** revealed 21 carbon signals, which were ascribed to three methyls (δ 21.3, 21.4, and 51.4), five methylenes (δ 18.4, 19.0, 36.8, 38.0, and 40.8), eight methines (δ 48.6, 55.1, 63.8, 110.8, 133.5, 138.6, 143.0, and 205.8), and five quaternary carbons (δ 37.0, 39.7, 124.4, 140.9, and 167.0). Assignments of the ^{1}H and ^{13}C signals of **2** by 2D-NMR spectra (Fig. 1 and Table 1) showed that **2** was an analogue of divinatorin D (**1**) in which the carbon at the C-17 position was oxidized to an aldehyde. The relative stereochemistry of the aldehyde at the C-8 position was assigned as α-orientation, based on NOESY cross-peaks between H-20 to H-17 and H-20 to H-19. On the basis of these data, the structure of **2** was elucidated as divinatorin E.

Compound **3** was isolated as a colorless semi-solid, $[\alpha]_D^{23}$ +180° (*c* 0.05, $CHCl_3$). Its molecular formula was deduced as $C_{23}H_{26}O_8$ by HR ESI-MS. The ^{1}H NMR spectrum displayed signals of three tertiary methyls (δ 1.27, 1.78, and 2.14), a methoxyl group (δ 3.84), two oxygenated methines (δ 5.51 and 5.80), and four olefinic protons (δ 6.41 × 2, 7.42, and 7.44), while the ^{13}C NMR spectrum of **3** showed 23 carbon signals, which were ascribed to 3 × CH_3, 1 × OCH_3, 3 × CH_2, 8 × CH, four quaternary carbons, and four carbonyl carbons. These data were similar to those of salvinorin D (**9**) isolated from the same extract. Comparison of the ^{1}H and ^{13}C NMR data between **3** and **9**[23] suggested that the two compounds differ in the A ring moiety. According to the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra (Fig. 1 and Table 1), it was confirmed that the hydroxyl group at C-2 in **9** was replaced by a carbonyl group (δ 192.9) in **3**. The relative stereochemistry of H-1 was assigned as β-orientation, based on NOESY cross-peaks between H-10 to H-1 and H-10 to H-8. From these data, the structure of **3** was determined as salvinorin G.

The affinities of the isolated compounds for the KOR were determined by competitive inhibition of [^{3}H]diprenorphine (0.2–0.4 nM) binding to the human KOR (hKOR) stably expressed in Chinese hamster ovary (CHO) cells as reported previously.[19] All compounds were initially screened to 10 μM. Only compounds **1**, **3**, **6**, and **7** at 10 μM inhibited [^{3}H]diprenorphine binding to the hKOR by more than 50%. Binding experiments were then carried out with a range of concentrations of the four compounds and the K_i value, a measure of binding affinity, of each compound for the hKOR was determined (Table 2). [^{35}S]GTγPS binding to G proteins in membranes, a direct measure of KOR activation, was used for the determination of potencies and

Table 2. K_i, EC_{50}, and E_{max} values of test compounds for the human κ opioid receptor

Compound	K_i (nM)	EC_{50} (nM)	E_{max} (%)
1	230 ± 21	359 ± 17	103
2	>10,000	NA	NA
3	418 ± 117	NA	NA
4	>10,000	NA	NA
5	>10,000	NA	NA
6	1.0 ± 0.1	3.1 ± 1.0	112
7	65.9 ± 8.6	172 ± 22	111
8	>10,000	NA	NA
9	>10,000	NA	NA
10	>10,000	NA	NA
11	>10,000	NA	NA
12	>10,000	NA	NA
13	>10,000	NA	NA
U50,488H	0.6 ± 0.04	3.9 ± 2.0	100

NA, EC_{50} was not determined due to low affinities.

efficacies of these compounds. Their EC_{50} and E_{max} values were calculated from the dose–response curves (Table 2).

Among the compounds, salvinorin A (**6**) has the highest affinity (K_i = 1.0 ± 0.1 nM) and is the most potent KOR agonist, comparable to U50,488H. Salvinorin B (**7**) has modest affinity for the KOR (K_i = 65.9 ± 8.6 nM) and is a low-potency full agonist. Our results are different from those of Chavkin et al.,[17] who reported that the K_i values of salvinorin A and salvinorin B for the KOR were 18.74 and >10,000 nM, respectively. Roth et al.[15] also reported a K_i value of 16 nM for salvinorin A. The discrepancy may result from the different radiolabeled ligands used in the studies, [^{3}H]bremazocine in the study of Roth et al.[15] and Chavkin et al.[17] and [^{3}H]diprenorphine in ours. Rusovici et al.[18] have demonstrated that bremazocine and U69,593 bind to low and high affinity states, respectively, of the human KOR expressed in CHO cells. [^{3}H]diprenorphine, an antagonist, in theory, binds to both states. Under our binding conditions, U50,488H showed a K_i value of 0.6 nM in competitive inhibition of [^{3}H]diprenorphine binding, consistent with the notion that [^{3}H]diprenorphine binds to at least the high-affinity state. In addition, diprenorphine and bremazocine, with different structures, most likely have distinct contact points within the binding pocket of KOR, thus salvinorin A and B may have differential abilities to displace these two ligands. Moreover, Chavkin et al.[17] included 150 mM NaCl in their binding buffer, whereas we did not. NaCl may reduce binding affinities of salvinorin A and salvinorin B. Chavkin et al.[17] reported that salvinorin B did not inhibit forskolin-stimulated adenylyl cyclase activity. We demonstrated that the EC_{50} value of salvinorin B in promoting [^{35}S]GTPγS binding was 172 nM. The difference is possibly due to the different functional assays used in the two studies. [^{35}S]GTPγS binding assay reflected the direct activation of G protein upon receptor activation, while adenylyl cyclase inhibition, mediated by the G_i protein, involved more downstream effectors. Regardless of the differences in absolute K_i and EC_{50} values, our study and that of Chavkin et al.[17] are in good agreement that salvinorin B has much lower affinity and potency for the KOR than salvinorin A.

Comparison between salvinorin A and salvinorin B indicates that acetyl group at the C-2 position is essential for high affinity and potency for the KOR. In general, C-ring opened compounds such as **1**, **2**, **4**, and **5** showed reduced KOR binding affinities. Furthermore, compounds with an unsaturated bond between C-3 and C-4, such as **3**, **8**, **9**, **10**, and **11**, did not improve binding activity, while compound **3** having a C-2 carbonyl group showed a modest binding affinity.

Since three types of opioid receptors shared high sequence homology, the binding affinities of the compounds **1**, **3**, **6**, and **7** were also determined for δ and μ opioid receptors stably expressed in CHO cells. Interestingly, **1**, with low affinity for KOR (K_i = 230 ± 21 nM), also showed modest affinity for δ opioid receptor (K_i = 625 ± 42 nM), but not for μ opioid receptor (K_i > 10,000 nM). All other compounds did not show appreciable binding to δ or μ opioid receptors at 10 μM.

Our findings provided more information on structure–activity relationships of salvinorin A and determination of pharmacophores for the hKOR.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with an AUTOPOL III digital polarimeter. NMR spectra were recorded on a Varian VXR300 spectrometer with TMS as the internal standard. EI-MS spectra were obtained on a HP5972 Series Mass spectrometer. ESI-TOFMS spectra were measured on a Micromass Plateform II mass spectrometer. Activated Carbon (Aldrich, USA), Celite 545 (Acros Organics, USA), and Silica Gel (Fisher Scientific, USA) were used for column chromatography. TLC was performed on precoated silica gel 60 F254 plates (Merck, Germany).

3.2. Plant material

The dried leaves of *S. divinorum* were purchased in August 2003 from Salvia Space (www.salviaspace.com). A voucher specimen (MCL-2003-09) has been deposited in the Bio-Organic and Natural Products Laboratory, McLean Hospital, Belmont, MA.

3.3. Extraction and isolation

The dried leaves of *S. divinorum* (4.6 kg) were powdered and extracted three times with hexane (25 L each time) at room temperature (25 °C) overnight, and the hexane solution was evaporated in vacuo to give a residue (70 g). The acetone and methanol extracts (330 and 290 g) were obtained by the same procedure. The acetone extract was fractioned by flash column chromatography with an equal mixture of activated carbon and Celite 545 (total 3000 g). The column was eluted with

acetone and hexane to give a pale yellowish solid (58 g). Recrystallizations from acetone and methanol gave pure salvinorin A (10 g). The mother liquor was chromatographed over silica gel (300 g) using chloroform with increasing amounts of acetone (20:1, 10:1, 5:1, 2.5:1, and 1:1) to give five fractions A–E. Fraction A (9.0 g), initially eluted from chloroform–acetone (20:1), was further purified by silica gel column (200 g), with a gradient of hexane and ethyl acetate (15:1–1:1) to afford fractions 1–108 (150 mL/fraction). The combined fractions were further purified by silica gel column using methylene chloride–ethyl acetate (20:1–10:1) or hexane–ethyl acetate (5:1–2:1) as the solvent system to give salvinorin-A (**6**, 2.5 g), -B (**7**, 270 mg), -C (**8**, 300 mg), -D (**9**, 18 mg), -E (**10**, 12 mg), -F (**11**, 6 mg), and -G (**3**, 8 mg), divinatorin-C (**4**, 20 mg), -D (**1**, 15 mg), and -E (**2**, 10 mg), hardwickiic acid (**5**, 25 mg), presqualene alcohol (**12**, 275 mg), and (*E*)-phytol (**13**, 120 mg).

3.4. Divinatorin D (1)

Semi-solid; $[\alpha]_D^{23}$ −172° (*c* 0.10, $CHCl_3$); ^1H NMR and ^{13}C NMR data, see Table 1; EI-MS *m/z*: 404 $[M]^+$ (2.3), 373 (2.1), 357 (12.3), 339 (7.2), 297 (3.8), 249 (25.8), 231 (11.1), 261 (16.0), 217 (25.5), 199 (25.9), 171 (16.1), 95 (100); HR EI-MS: *m/z* 404.2187 (M^+, calcd for $C_{23}H_{32}O_6$ 404.2199).

3.5. Divinatorin E (2)

Semi-solid; $[\alpha]_D^{23}$ −58° (*c* 0.05, $CHCl_3$); ^1H NMR and ^{13}C NMR data, see Table 1; EI-MS *m/z*: 360 $[M]^+$ (2.1), 342 (1.0), 329 (2.5), 313 (6.4), 295 (6.0), 285 (9.9), 265 (9.7), 235 (9.7), 205 (13.5), 187 (36.6), 159 (31.9), 96 (100); HR EI-MS: *m/z* 360.1927 (M^+, calcd for $C_{21}H_{28}O_5$ 360.1937).

3.6. Salvinorin G (3)

Semi-solid; $[\alpha]_D^{23}$ +180° (*c* 0.05, $CHCl_3$); ^1H NMR and ^{13}C NMR data, see Table 1; ESI-MS *m/z*: 431 $[M+1]^+$; HR ESI-MS: *m/z* 431.1713 (M^+, calcd for $C_{23}H_{26}O_8$ 431.1706).

3.7. Competitive inhibition of [^{3}H]diprenorphine binding to opioid receptors

The assay was carried out as described previously.[19] The K_i value of each compound was calculated from the competitive inhibition curves using the Prism 3.0 program (GraphPad Software Inc., San Diego, CA).

3.8. [^{35}S]GTPγS binding

The assay was performed as previously detailed.[19] EC_{50} and E_{max} values of each compound were obtained from dose–response curves by sigmoidal curve fitting using the Prism 3.0 program (GraphPad Software Inc., San Diego, CA). The EC_{50} and E_{max} values (relative to U50,488H) represent the potency and maximal effect of each compound, respectively, to enhance [^{35}S]GTPγS binding to G proteins upon activation of the human KOR.

Acknowledgments

The author thanks Drs. Puling Wang and Qing Liao, Department of Chemistry and Chemical Biology, Harvard University, for MS determination, Dr. Ruicao Shen for help with optical rotation measurements, and Dr. Cecile Beguin for procurement of the *S. divinorum* leaves.

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