JOURNAL OF NATURAL PRODUCTS

Article

Cite This: J. Nat. Prod. XXXX, XXX, XXX−XXX

pubs.acs.org/jnp

Ferulic Acid Esters and Withanolides: In Search of *Withania somnifera* $GABA_A$ Receptor Modulators

Vijay P. Sonar,[†,‡] Benedetta Fois,[†] Simona Distinto,[†] Elias Maccioni,[†] Rita Meleddu,[†] Filippo Cottiglia,[*,†] Elio Acquas,[†,§] Sanjay Kasture,[⊥] Costantino Floris,[∥] Daniele Colombo,[†] Claudia Sissi,[#] Enrico Sanna,[†,§] and Giuseppe Talani[¶]

[†]Department of Life and Environmental Sciences, University of Cagliari, 09124 Cagliari, Italy

[‡]R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, 425404 India

[§]Centre of Excellence on Neurobiology of Addiction, University of Cagliari, 09042 Cagliari, Italy

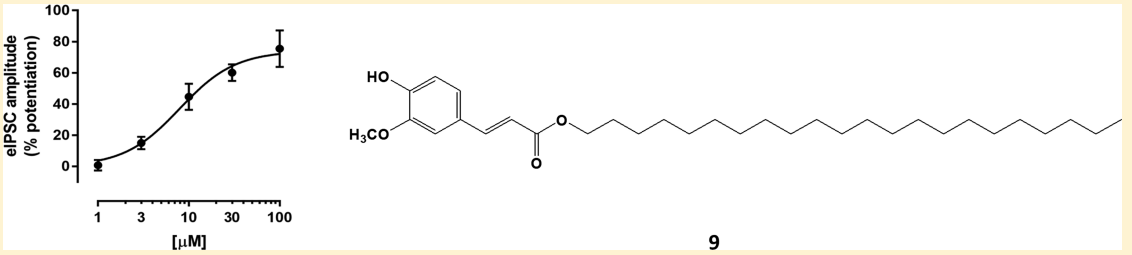
[⊥]Department of Pharmacology, Sanjivani College of Pharmaceutical Education & Research, Kopargaon, 423603 India

[∥]Department of Chemical and Geological Sciences, University of Cagliari, 09042 Monserrato, Italy

[#]Department of Pharmaceutical and Pharmacological Sciences, University of Padova, 35131 Padova, Italy

[¶]National Research Council (CNR), Institute of Neuroscience, 09042 Monserrato, Italy

Supporting Information



ABSTRACT: Nine compounds, including two undescribed withanolides, withasomniferolides A and B (**1** and **2**), three known withanolides (**3**−**5**), a ferulic acid dimeric ester (**6**), and an inseparable mixture of three long alkyl chain ferulic acid esters (**7**−**9**), were isolated from a $GABA_A$ receptor positive activator methanol extract of the roots of *Withania somnifera*. The structures of the isolated compounds were elucidated based on NMR, MS, and ECD data analysis. In order to bioassay the single ferulic acid derivatives, compounds **6**−**9** were also synthesized. The most active compound, docosanyl ferulate (**9**), was able to enhance the $GABA_A$ receptor inhibitory postsynaptic currents with an IC_{50} value of 7.9 μM. These results, by showing an ability to modulate the $GABA_A$ receptor function, cast fresh light on the biological activities of the secondary metabolites of *W. somnifera* roots.

Withania somnifera (L.) Dunal, also known as "Ashwagandha" or Indian ginseng, is a green shrub belonging to the family Solanaceae. Due to its adaptogenic properties known since antiquity, in Ayurveda *W. somnifera* holds a place similar to ginseng in Chinese medicine, as being capable of imparting long life, youthful vigor, and intellectual prowess. Accordingly, *W. somnifera* is an ingredient of many traditional medicine preparations.[1] The plant is used to treat various neurological disorders, geriatric debilities, insomnia, anxiety, stress, and behavior-related problems.[2,3] Both clinical[4−6] and animal[7−11] studies have supported the traditional use of "Ashwagandha" as an antistress and anxiolytic remedy. Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central and peripheral nervous system, and the GABAergic system in the mammalian brain plays a critical role in the regulation of anxiety and stress. In addition, ionotropic GABA type A receptors (GABAAR) are the most important target for a variety of anxiolytic and anticonvulsant drugs such as benzodiazepines. Interestingly, the anxiolytic effect of *W. somnifera* root extracts has been often attributed to its main interaction with the $GABA_A$ receptor channel complex. In particular, Mehta et al.[12] reported for the first time that, in a radioligand binding assay, a methanol extract of the roots of *W. somnifera* was able to interact with the GABAergic system, binding the GABAAR site and thus possessing a GABA-mimetic activity. More recently, the GABA-mimetic activity of a methanol extract of the roots of *W. somnifera* was confirmed in whole-cell patch-clamp recordings in gonadotropin-releasing-hormone (GnRH) neurons. Indeed, this extract was shown to affect the firing rate of these neurons by acting on GABAAR.[13]

As a part of an ongoing research program focused on the elucidation of the mechanism(s) by which the methanol

Received: December 4, 2018

DOI: 10.1021/acs.jnatprod.8b01023
J. Nat. Prod. XXXX, XXX, XXX−XXX

extract of the roots of *W. somnifera* modulates certain effects induced by morphine (thus prolonging analgesia and suppressing hyperalgesia), the affinities of this extract against several receptors involved in pain transduction signaling (μ, δ, κ, CB_1, CB_2, $GABA_A$, $GABA_B$, NMDA, α_2, 5-HT_{2A}) have been studied.[14,15] This extract showed affinity against the GABAAR with a K_i of 13 μg/mL, confirming the data previously reported by other authors. Surprisingly, despite various studies that demonstrated clearly the affinity of the methanol extract of the roots of *W. somnifera* against GABAAR, the compounds responsible for this effect have not been identified yet. Moreover, when withaferin A and withanolide A, two of the main constituents of the roots of *W. somnifera*, were tested against the native rat brain GABAAR expressed in *Xenopus* oocytes, neither compound was able to activate the GABAAR complex.[16]

RESULTS AND DISCUSSION

Compound Isolation and Structure Elucidation. The methanol extract of the roots of *W. somnifera*, which exhibited modulatory activity at the GABAA receptor, was treated with CH_2Cl_2 and the soluble portion investigated phytochemically. Investigation of the CH_2Cl_2 fraction led to the isolation of two previously undescribed (**1** and **2**) and three known withanolides (**3**–**5**) together with four ferulic acid esters (**6**–**9**). In order to evaluate the potential modulatory activity of all the isolated compounds on the function of GABAAR, GABA-induced inhibitory postsynaptic currents (IPSCs) were evaluated in whole-cell patch-clamp recordings in rat hippocampal slices.

The HRESIMS of compound **1** showed a molecular ion at *m/z* 437.2669 (calcd 437.2684). This molecular mass in combination with ^{1}H and ^{13}C NMR data allowed the molecular formula to be established as $C_{28}H_{36}O_4$. The IR spectrum of **1** revealed the presence of hydroxy (3399 cm^{-1}), α,β-unsaturated δ-lactone, and α,β-unsaturated carbonyl (1690, 1652 cm^{-1}) groups. The UV spectrum showed an absorption maximum at 222 nm, supporting the presence of an α,β-unsaturated carbonyl moiety.

In the ^{1}H NMR spectrum of compound **1**, four methyl groups at δ_H 0.78 (3H, s), 1.00 (3H, d, J = 6.5 Hz), 1.23 (3H, s), and 2.00 (3H, s) could be detected besides a group of CH_2 and CH signals between 1 and 2 ppm. In the same spectrum, five olefinic protons at δ_H 6.96 (1H, dd, J = 10.2 Hz), 6.13 (1H, dd, J = 10, 2.5 Hz), 5.93 (1H, d, J = 5.5 Hz), 5.92 (1H, d, J = 9.5 Hz), and 5.81 (1H, dd, J = 10, 2 Hz), an oxymethylene group at δ_H 4.37 (1H, d, J = 12.5 Hz) and 4.38 (1H, d, J = 12.5 Hz), and an oxymethine proton at δ_H 4.46 (1H, m) were also present. With the aid of HSQC experiment, all the observed protons were assigned to their respective carbons. In the ^{1}H–^{1}H COSY spectrum (Figure 1), the proton signal at 5.93 ppm correlated with that at δ_H 6.96, which in turn, correlated with the proton at 5.92 ppm, while the methyl at 1.02 ppm showed a cross-peak with the CH signal at δ_H 2.06. In the HMBC spectrum (Figure 1), the long-range correlations between the oxymethylene protons at δ_H 4.37 (δ_C 57.3) and the carbons at δ_C 166.8, 152.6, and 125.7 and those of the methyl at δ_H 2.04 (δ_C 20.0) with the carbons at δ_C 152.6, 125.7, and 29.9 suggested the presence of a lactone ring characteristic of a withanolide. Thus, in the same experiment, it could be noted that the lactone ring is linked to the cyclopentane ring of the withanolide through a methine group, as shown by the cross-peaks between the methyl at δ_H 1.02 (d, J = 6.5 Hz) and the carbons at δ_C 38.9 (C-20), 52.0 (C-17), and 78.8 (C-22). Additional correlations between the olefinic proton at δ_H 6.96 (δ_C 140.4) and the carbons at δ_C 204.8 (C-1) and 155.4 (C-5), between the olefinic proton at δ_H 6.13 (δ_C 127.5) and the carbons at δ_C 155.4 (C-5), 117.2 (C-4), 50.8 (C-10), and 37.9 (C-8), and between the methyl at δ_H 1.23 (δ_C 19.9) and C-1 (δ_C 204.8), C-10 (δ_C 50.8), and C-9 (δ_C 47.1) permitted the 8,9-dihydronaphthalene-1-one nucleus of the A and B fused rings to be defined. ROESY experiments and analysis of scalar ($^3J_{H-H}$) coupling of the protons were used to determine the stereochemistry for most of the molecule. Strong ROESY cross-peaks from H-8 (δ_H 2.33) to H-18 (δ_H 0.82) and H-19 (δ_H 1.26) and absent cross-peak from H-18 to H-17 implied that H-17 is on the other side of the molecule with respect to H-8, H-18, and H-19 (Figure 1). The configuration at C-22 could be presumed as *R*, as the literature indicated that an α-oriented H at C-22 would give rise to $J_{22,23}$ values between 0.5 and 7 and 9–13.8 Hz, whereas the β-oriented form would exhibit values between 2.5-7 and 2–5 Hz. The observed coupling constants for the proton at C-22 (J = 3.5 and 13 Hz) conformed with the former limits.[17] The electronic circular dichroism (ECD) spectrum of **1**

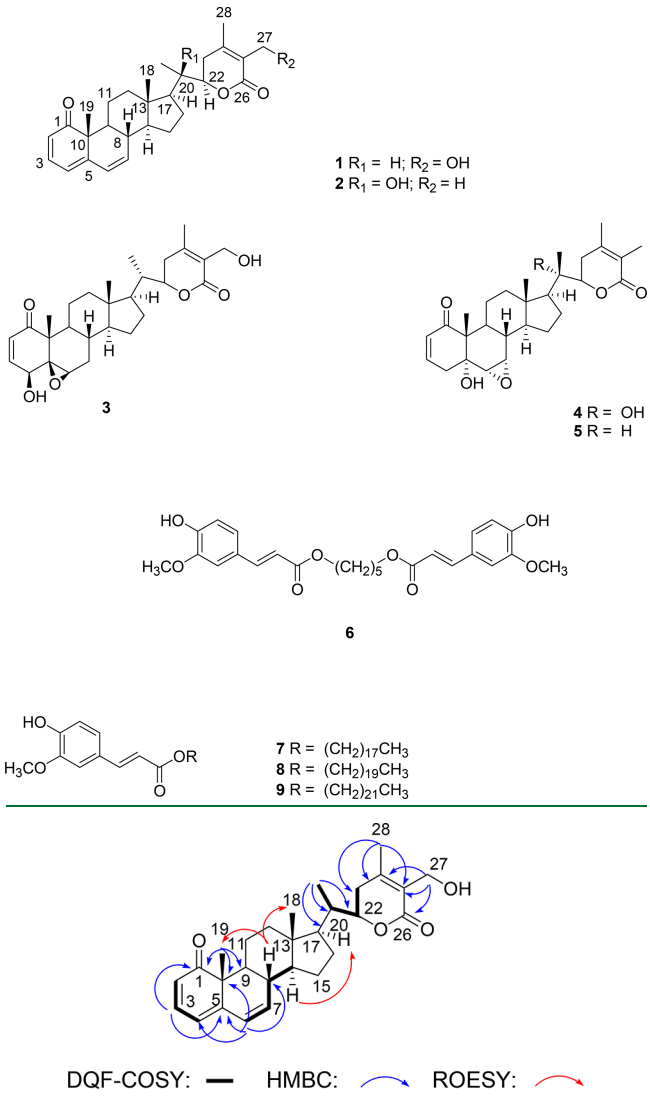


Figure 1. Main HMBC, DQF-COSY, and ROESY correlations of withasomniferolide A (**1**).

DOI: 10.1021/acs.jnatprod.8b01023
J. Nat. Prod. XXXX, XXX, XXX–XXX

exhibited a negative Cotton effect at 350 nm, confirming the configuration and the *trans* linkage of the A and B rings,[18] and a positive Cotton effect at 251 nm based on the n → π* transition of an α,β-unsaturated δ-lactone, confirming the *R* configuration of C-22.[18] The configuration at C-20 was difficult to establish from the ROESY spectrum due to the free rotation of the C-17−C-20 and C-20−C-22 bonds. However, it was determined as *R* on the basis of biogenetic arguments because all the reported withanolides unsubstituted at C-20 have the same configuration.[19] A detailed study of DQF-COSY, HSQC, and HMBC experiments allowed the complete assignment of all NMR spectroscopic data and the assignment of compound **1** as (20*R*,22*R*)-1-oxo-27-hydroxywitha-2,4,6-trienolide. Since compound **1** is a previously undescribed molecule, it was named withasomniferolide A.

Compound **2** showed a pseudomolecular ion peak at *m*/*z* 437.2683 (calcd 437.2684) in the HRESIMS, accounting for the elemental composition, $C_{28}H_{36}O_4$. The IR spectrum of **2** revealed the presence of hydroxy group (3366 cm^{-1}), α,β-unsaturated δ-lactone, and α,β-unsaturated carbonyl (1705, 1626 cm^{-1}) functionalities. The downfield region ($5.7 < \delta_H < 7$) of the ^1H NMR spectrum of compound **2** was superimposable on that of **1**, suggesting the presence of a 8,9-dihydronaphthalene-1-one moiety. The major significant differences between the spectra of compounds **2** and **1** were the upfield shift of the oxygenated methine at δ_H 4.21 (1H, dd, *J* = 3.2, 13.2 Hz) and the absence of the oxymethylene protons at C-27 (δ_H 4.37 and 4.38 in the spectrum of **1**) (Table 1). Another difference between the two ^1H NMR spectra was the number of methyl groups, with four in compound **1** and five in compound **2**. DQF-COSY, HSQC, and HMBC experiments revealed that rings A−D of compound **2** are consistent with those of withasomniferolide A. The HMBC spectrum showed correlations from the methyl protons at 1.30 ppm (δ_C 20.9) to the quaternary carbon at δ_C 75.1 and to the methine carbons at δ_C 54.6 and 80.9 as well as from methyl protons at δ_H 1.95 (δ_C 20.5) to carbons at δ_C 31.6, 148.8, and 122.0 and from the CH_3 at δ_H 1.89 (δ_C12.5) to carbons at δ_C 148.8, 122.0, and 166.0. These connectivities led to the conclusion that a methyl group is located at the C-25 position in compound **2**. Further HMBC cross-peaks between H-22 (δ_H 4.21) and C-21 (δ_C 20.9), C-20 (δ_C 75.1), and C-24 (δ_C 148.8) and between H-21 (δ_H 1.30) and C-22 (δ_C80.9), C-20, and C-17 (δ_C 54.6) revealed that C-20 is a quaternary oxygenated carbon. The location of the OH group at C-20 was confirmed by the shift of the γ-positioned C-16 (7 ppm decrease) due to the γ-gauche effect,[20] when compared with that of C-20 in unsubstituted compound **1**. Thus, compound **2** is an isomer of withasomniferolide A and differs from the latter only for the translocation of the OH group from position C-27 to C-20. Furthermore, ROESY cross-peaks confirmed the same configuration assigned for withasomniferolide A. The ECD spectrum of **2** exhibited a negative Cotton effect at 350 nm, confirming the configuration and the *trans* linkage of the A and B rings,[18] and a positive Cotton effect at 251 nm, confirming the *R* configuration of C-22.[18] Therefore, the structure of compound **2** (withasomniferolide B) was determined as (22*R*)-1-oxo-20-hydroxywitha-2,4,6-trienolide.

The ^1H NMR spectrum of compound **6** showed typical signals of a ferulic acid derivative at δ_H 7.61 (d, *J* = 16 Hz, 1H), 7.06 (dd, *J* = 1.5, 8 Hz, 1H), 7.02 (d, *J* = 1.5 Hz, 1H), 6.90 (d, *J* = 8.0 Hz, 1H), 6.28 (d, *J* = 16 Hz, 1H), and 3.91 (s, 3H). The chemical shift values at δ_C 167.3 and 64.2 ppm displayed

Table 1. ^1H NMR and ^{13}C NMR Spectroscopic Data for Compounds 1 and 2 ($CDCl_3$, δ in ppm)

	compound **1**		compound **2**	
position	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	204.8, C		204.8, C	
2	125.5, CH	5.92, d (9.5)	125.4, CH	5.91, d (9.5)
3	140.4, CH	6.96, dd (9.5, 5.5)	140.4, CH	6.96, dd (9.5, 5.5)
4	117.2, CH	5.93, d (5.5)	117.1, CH	5.93, d (5.5)
5	155.4, C		155.5, C	
6	127.5, CH	6.13, dd (10, 2.5)	127.4, CH	6.13, dd (10, 2.5)
7	134.7, CH	5.81, dd (10, 2)	134.9, CH	5.83, dd (10, 2)
8	37.9,CH	2.33, m	37.3, CH	2.31, m
9	47.1, CH	1.60, m	47.1, CH	1.55, m
10	50.8, C		51.0, C	
11	39.8, CH_2	Ha: 2.05, m[a]	40.2, CH_2	Ha: 2.06, m[a]
		Hb: 1.16, m		Hb: 1.19 m
12	27.3, CH_2	Ha: 1.69, m	22.4, CH_2	2.23, m
		Hb: 1.35, m		
13	43.9, C		44.1, C	
14	53.9, CH	1.21, m	54.3, CH	1.23, m
15	23.8, CH_2	1.80, m	23.5, CH_2	1.79, m
16	29.8, CH_2	1.25, m	21.9, CH_2	1.98, m[a]
17	52.0, CH	1.16, m	54.6, CH	1.49, m
18	11.8, CH_3	0.82, s	13.6, CH_3	0.98, s
19	19.9, CH_3	1.23, s	20.0, CH_3	1.26, s
20	38.9, CH	2.06, m[a]	75.1, C	
21	13.3, CH_3	1.02, d (6.5)	20.9, CH_3	1.30, s
22	78.8, CH	4.46, m	80.9, CH	4.21, dd (3.2, 13.2)
23	29.9, CH_2	Ha: 2.50, m	31.6, CH_2	Ha: 2.39, m
		Hb: 1.99, m		Hb: 2.05, m[a]
24	152.6, C		148.8, C	
25	125.7, C		122.0, C	
26	167.0, C		166.0, C	
27	57.5, CH_2	Ha: 4.38, d (12.5)	20.5, CH_3	1.95, s
		Hb: 4.37, d (12.5)		
28	20.0, CH_3	2.04, s[a]	12.5, CH_3	1.89, s

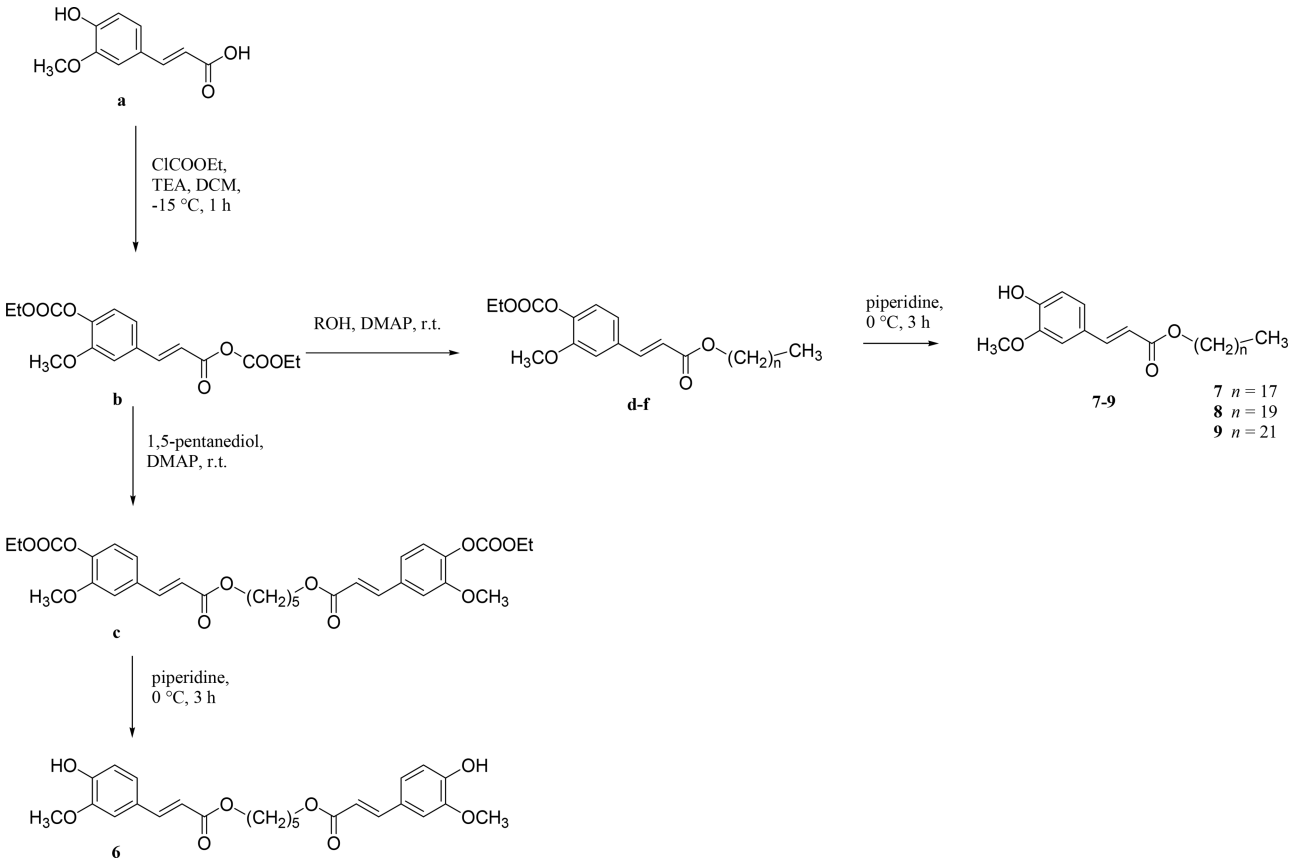
[a]Signals were overlapped.

in the ^{13}C NMR spectrum of compound **6**, besides the long-range correlations of the methylene protons at δ_C 64.2, with the carbons at δ_C 167.3, 28.5, and 26.5 ppm observed in the HMBC spectrum, suggested the presence of a ferulic acid alkyl ester. However, in the ^1H NMR spectrum a terminal methyl group or a further oxymethylene moiety could not be detected, suggesting the presence of a ferulate dimeric derivative. The ESIMS spectrum showed a molecular ion peak at *m*/*z* 455 $[M - H]^-$ and a fragmentation ion at *m*/*z* 279 $[M - C_{10}H_9O_3]^-$ (Figure S23, Supporting Information). This is in accord with the molecular formula $C_{25}H_{28}O_8$, and therefore compound **6** was identified as 1,5-di-*O*-feruloylpentanediol. The ^1H and ^{13}C NMR values as well as the MS data are congruent with literature data.[21] In order to confirm the structure and to obtain more compound for the biological screenings, compound **6** was also synthesized (see the next paragraph). Interestingly, although 1,5-di-*O*-feruloylpentanediol (**6**) has been obtained by synthesis,[21] it has been isolated for the first time as a natural product in the present study.

Octadecyl ferulate (**7**), eicosanyl ferulate (**8**), and docosanyl ferulate (**9**) were obtained as an inseparable mixture. The ^1H NMR spectrum of the mixture matched with a long alkyl chain ferulate. The ESIMS (negative mode) revealed three ion peaks

DOI: 10.1021/acs.jnatprod.8b01023
J. Nat. Prod. XXXX, XXX, XXX−XXX

Scheme 1. Synthetic Pathway to Compounds 6−9



at m/z 501, 473, and 445 (Figure S16, Supporting Information). MS/MS spectra of the three ion peaks, each showing a loss of a methyl group (Figures S24−27, Supporting Information), clearly demonstrated that the peaks at m/z 473 and 445 did not originate from that at m/z 501. Therefore, the compound was a mixture of three long-chain alkyl ferulates, differing from each other only by the number of methylene groups and was identified as a mixture of octadecyl ferulate (**7**), eicosanyl ferulate (**8**), and docosanyl ferulate (**9**). Their structures were confirmed by comparison of their spectroscopic data[22] and by synthesis (see Scheme 1). These three ferulates have been isolated for the first time from this plant. Previous studies reported the modest antioxidant activity of compound **7**[23] and the antiproliferative activity of compound **8** toward U87-MG and SW620 cells.[24] On the contrary, no biological study, to the best of our knowledge, is available for compound **9**. The spectroscopic (^{1}H and ^{13}C NMR, UV, and MS) and physical data (melting point, optical rotation) of the known compounds withaferin A (**3**),[25] withanolide A (**4**),[26] and withanolide B (**5**)[27] were in agreement with the literature data.

Synthesis of 1,5-Di-*O*-feruloylpentanediol. The synthesis of 1,5-di-*O*-feruloylpentanediol (**6**) was carried out according to a previously described procedure,[28] with some minor modifications, as illustrated in Scheme 1.

Synthesis of Compounds 7−9. The synthesis of each of octadecyl ferulate (**7**), eicosanyl ferulate (**8**), and docosanyl ferulate (**9**) was carried out using the same procedure as for compound **6**, but with a different molar ratio between alcohols and mixed anhydride (see Experimental Section) (Scheme 1).

Modulatory Effects of Compounds 1−9 at the GABAAR. In order to evaluate the potential modulatory activity of the methanol extract of *W. somnifera* and, comparatively, that of compounds **1**−**9** on the function of GABAAR, inhibitory postsynaptic currents were evoked in single voltage-clamped (−65 mV) granule cells of the dentate gyrus (DG), present in acute rat hippocampal slices. To this end, GABAergic afferents, coming from surrounding inhibitory interneurons and projecting mainly to the cell body of granule neurons, were stimulated electrically, and the resulting release of GABA generated transient IPSCs due to the activation of postsynaptic GABAAR. For control responses, the intensity of the stimulation was set as to evoke IPSCs for which the amplitude was ∼30% of the maximal response and was determined at the beginning of each recording. As expected, bath application of the GABAAR-selective antagonist bicuculline (20 μM) suppressed the evoked IPSCs, confirming that they were in fact mediated by activation of this receptor (data not shown). In different DG granule cells, IPSCs were recorded before and after perfusion of either 400 μg/mL of the extract or 10 μM of each of the pure compounds (**1**−**9**). In the presence of the extract, IPSC amplitude was significantly enhanced (55.1 ± 16.1%; $p < 0.05$) with respect to the control response (Figure 2a,b). The amplitude of IPSCs was also increased in the presence of 10 μM docosanyl ferulate (**9**) (48.2 ± 16.7%; $p < 0.05$) and withanolide B (**5**) (29.4 ± 5.1%; $p < 0.005$). Conversely, at the same concentration, withasomniferolide B (**2**), octadecyl ferulate (**7**), eicosanyl ferulate (**8**), and withanolide A (**4**) reduced IPSC amplitude by 28.6 ± 8.9% ($p < 0.05$), 35.8 ± 8.8% ($p < 0.05$), 36.5 ± 6.9% ($p < 0.05$), and 20.8 ± 4.6% ($p < 0.05$), respectively. Compounds **1**,

DOI: 10.1021/acs.jnatprod.8b01023
J. Nat. Prod. XXXX, XXX, XXX−XXX

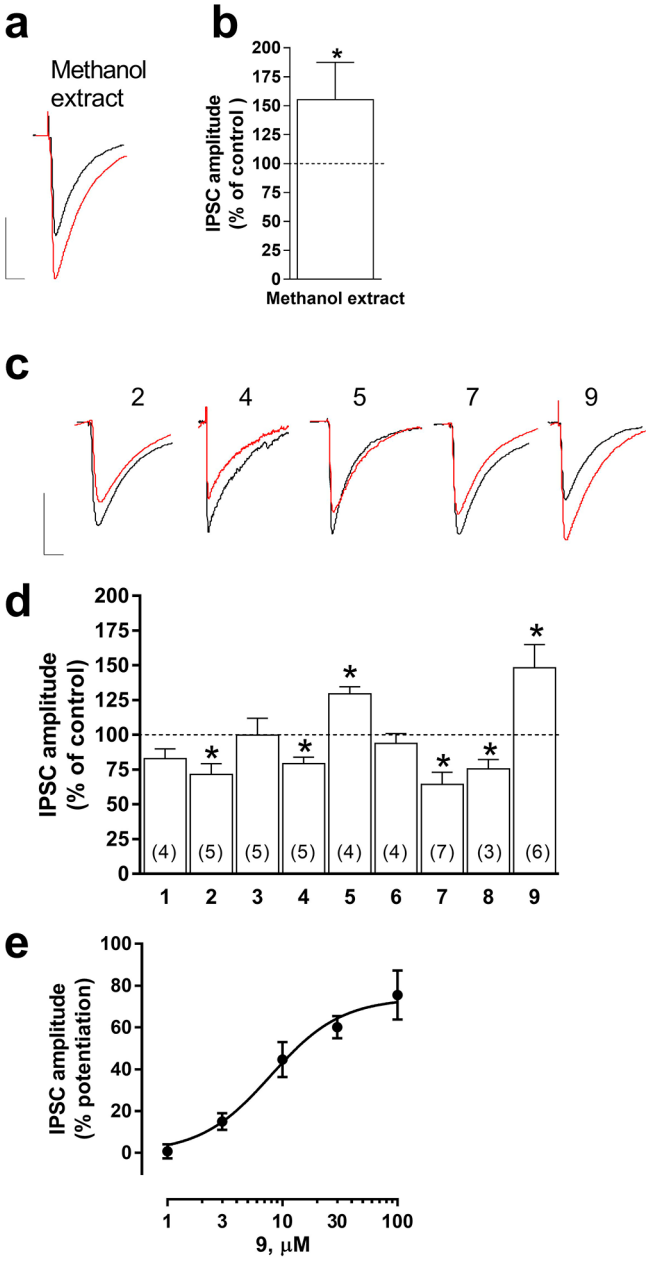


Figure 2. Effects of the methanol extract of *W. somnifera* and compounds **1**–**9** on IPSCs recorded from rat dentate gyrus hippocampal neurons. (a, c) Representative traces of IPSCs recorded from a single granule neuron before (black trace) and during (red trace) the bath application of the extract (400 μg/mL) and compounds **1**–**9** (10 μM). Scale bar: 100 pA, 20 ms. Only compounds with a clear effect are reported. (b, d) The bar graph (d) shows the cumulative effect of the extract and the isolated compounds on their modulatory action of GABA-mediated IPSCs. Data are expressed as percent change from baseline and are means ± SEM. The number of cells analyzed is indicated by the number inside each bar. (e) Concentration–response curve of compound **9** on GABAAR-mediated currents (*$p < 0.05$ vs baseline).

3, and **6** were ineffective in altering IPSC amplitude (Figure 2c,d). None of these nine compounds had the ability, at the concentration of 10 μM, to alter the holding current in the absence of electrical stimulation, suggesting the lack of direct activation of GABAAR (data not shown). A concentration–response curve was established for compound **9** and resulted in a maximal potentiation of 74 ± 11.7% at 100 μM, with an EC_{50} value of 7.9 μM (Figure 2e).

Previous attempts to identify specific components present in root extracts of *W. somnifera* as responsible for their GABAergic activity proved unsuccessful: thus, for example, both withaferin A and withanolide A were devoid of any appreciable GABAergic activity.[16] However, in their work, Candelario et al.[16] evaluated only the direct, GABA-mimetic, effect of these two compounds but did not measure their potential modulatory action on GABAA receptor function. In the present investigation, while no direct GABA-like action of the test compounds (at 10 μM) could be observed, evidence for several compounds showing a differential modulatory activity of GABAAR function was found and may be responsible, at least in part, for the GABAergic activity of the methanol root extract. Interestingly, the occurrence of ferulic acid esters has been so far unreported in both leaves and roots of *W. somnifera*. This fact is probably due to the complex mixture of long-chain alkyl ferulates that precludes the identification of single metabolites. Among all nine compounds tested, docosanyl ferulate (**9**) was the most active, with an IC_{50} value of 7.9 μM (Figure 2d,e), while withanolide B (**5**) was less active. Interestingly, a structure–activity relationship of the alkyl ferulates (**7**–**9**) revealed that GABA-induced current modulation was strictly dependent on the length of the alkyl chain. In fact, eicosanyl ferulate (**8**), containing a medium size chain (C_{20}), was not able to alter the IPSCs amplitude, whereas the compound with the shorter chain (C_{18}) showed an effect on IPSCs' amplitude that mimics the pharmacological profile of inverse agonists[29] and opposite that observed with the longer chain compound, docosanyl ferulate (**9**) (C_{22}). Furthermore, the new withanolide **1** was devoid of any GABA-induced current modulation. The previously undescribed withanolides (**1**, **2**) contain a rare 2,4,6-trienone functionality that could originate from the extraction procedure that was carried out by using hot methanol. In fact, this extraction procedure could lead to opening the 5,6- or 6,7-epoxide ring that is present in most of the withanolides. The subsequent elimination of the hydroxy group would lead to the highly conjugated system present in compounds **1** and **2**.

In order to exclude the formation of artifacts during the isolation procedure, the dichloromethane-soluble portion of *W. somnifera* methanol extract initially was injected into the HPLC, using an RP-18 column. The HPLC chromatogram (Figure S30, Supporting Information) clearly showed both compounds to be present, although as minor components, in the commercial methanol extract. The next step was to verify the formation of artifacts during the extraction procedure. Withanolide A, the most abundant withanolide, containing a 5-hydroxy-6,7-epoxide group, in the presence of hot methanol and of a reducing agent, could eliminate two hydroxy groups, giving compound **2**. To this end, a solution of withanolide A in methanol was heated under reflux for 8 h in the absence or presence of (+)-glucose, as reducing agent. The choice of (+)-glucose is due to the fact that a methanol extract of *W. somnifera* roots contains high amounts of sugars and, among these, glucose.[30] Either with (+)-glucose or without, the expected compound **2** could not be observed, and withanolide A was found to be stable under these conditions. However, this procedure is not conclusive, and the possibility that compounds **1** and **2** may be artifacts produced during the extraction procedure, starting from minor compounds, cannot be excluded.

DOI: 10.1021/acs.jnatprod.8b01023
J. Nat. Prod. XXXX, XXX, XXX–XXX

Electrophysiological results suggest that the GABAergic effect of the methanol extract of *W. somnifera* may be important for the anxiolytic action.[31,32] Some compounds (**5**, **9**) showed an overall similar pharmacological profile to the extract (see Figure 2), while others (**2**, **4**, **7**, **8**) revealed an opposite effect, suggesting that the main pharmacological effect may mainly depend on compounds that are positive activators of GABAAR compared with those that decrease GABAAR activity. Overall, ex vivo data obtained demonstrated that different compounds obtained from the methanol extract of *W. somnifera* have pharmacological properties similar to other known GABAAR allosteric modulators, suggesting that they may represent potential natural anxiolytic drugs. Future work on these compounds will attempt to characterize the potential site of interaction at the GABAAR complex and to establish whether they possess any selectivity for a specific subpopulation of GABAAR, as in the case of different allosteric modulators of these receptors. This information could also help the design of more drug-like compounds. The main issue of the most active compound (**9**) is the high number of rotatable bonds and the low solubility (Qikprop results; Figure S28, Supporting Information). However, some chemical modification could overcome these limitations.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in $CHCl_3$ or MeOH at 25 °C using a PerkinElmer 241 polarimeter. Circular dichroism spectra were recorded on a JASCO J-810 spectropolarimeter equipped with a Peltier temperature controller using a 10 mm path-length cell. All measurements were performed in methanol at a compound concentration of 300 μM. Each reported spectrum represents the average of 3 scans recorded with 1 nm step resolution. Observed ECD signals were converted to molar ellipticity $[\Theta]$ = deg × cm^2 × $dmol^{-1}$. UV spectra were recorded on a GBC Cintra 5 spectrophotometer. IR spectra were performed with a Nicolet iS10 FTIR spectrometer (Thermo Fisher Scientific). NMR spectra of all isolated compounds were recorded at 25 °C on a Unity Inova 500NB high-resolution spectrometer (Agilent Technologies, CA, USA) operating at 500 MHz for ^1H and 100 MHz for ^{13}C, respectively. Compounds were measured in $CDCl_3$, and the spectra referenced against residual nondeuterated solvents. HRESIMS were measured on an Agilent 6520 time of flight (TOF) MS instrument, while ESIMS were measured on an AB Sciex 3200 QTrap LC-MS/MS triple quadrupole instrument. Column chromatography was carried out under TLC monitoring using silica gel (40−63 μm, Merck) and Sephadex LH-20 (25−100 μm, Pharmacia). For vacuum-liquid chromatography (VLC), silica gel (40−63 μm) (Merck) was used. TLC was performed on silica gel 60 F_{254} or RP-18 F_{254} (Merck). Semipreparative HPLC was conducted by means of a Varian 920 LH instrument fitted with an autosampler module with a 1000 μL loop. The peak purities were monitored using a dual-wavelength UV detector set at 254 and 366 nm. The columns were a 250 × 10 mm Spherisorb silica, particle size 5 μm (Waters), and a 250 × 10 mm Polaris C-18-A, particle size 5 μm (Varian).

Plant Material. The methanol extract of the roots of *W. somnifera* was kindly provided by Natural Remedies Pvt. Ltd., Bangalore, India. The roots of *W. somnifera* were collected in January 2011, at Bangalore, India. The plant material was identified by Prof. Meera Sumanth (Vishvesharayya College of Pharmacy, Bangalore India) and Dr. M. Deepak (Natural Remedies Pvt. Ltd.). A reference sample of the extract (WS 11003) was deposited at the Department of Life and Environmental Science, Drug Sciences Section, University of Cagliari. The dried and powered roots of *W. somnifera* (1 kg) were extracted with MeOH in a Soxhlet apparatus to give 220 g of dried extract. The extract was characterized by an HPLC-fingerprint analysis, as certified by Natural Remedies Pvt. Ltd., with identification of the main withanolides present in the extract (Figure S29, Supporting Information).

Extraction and Isolation. The methanol extract (20 g) was treated with CH_2Cl_2, and the soluble portion (1.2 g) was subjected to column chromatography (CC) over silica gel, eluted with toluene−EtOAc (9.5:0.5). Ten fractions (F1−F10) were obtained, and F8 (370 mg) was further purified by CC over silica gel, using *n*-hexane−EtOAc (4:6), to give nine subfractions (F8.1−F8.9). The *n*-hexane-soluble portion of F8.4 (75 mg) was chromatographed over Sephadex LH-20 (MeOH) to get a more pure fraction (F8.4.1), which was further purified by RP-HPLC using acetonitrile−H_2O (7:3, flow 2.5 mL/min) as eluent, to provide compound **1** (2.2 mg, t_R 12 min). Fraction F8.7 (19 mg) was chromatographed by RP-HPLC using acetonitrile−H_2O (7:3, flow 2.5 mL/min) to yield compound **2** (2.1 mg, t_R 9.2 min). Fraction F8.5 (9.6 mg) was a mixture of compounds **7**−**9**. Fraction F8.3 (20.1 mg) was purified by CC over Sephadex LH-20 (MeOH) to give compound **6** (6.9 mg). Fraction F8.6 (18 mg) was purified by RP-HPLC using acetonitrile−H_2O (6:4, flow 2.5 mL/min) to yield compound **5** (4.0 mg, t_R7.6 min). Fraction F9 was identified as compound **4** (32 mg). Fraction F10 (480 mg) was subjected to CC using *n*-hexane−EtOAc (6:4) as eluent to provide compound **3** (15.5 mg).

*Withasomniferolide A (**1**):* pale yellow powder; $[\alpha]^{25}_D$ +18.8 (*c* 0.05, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 220 (4.11), 350 (1.0) nm; ECD (300 μM, MeOH) λ ($\Delta\varepsilon$) 251 (+3920), 350 (−2670) nm; IR (KBr) ν_{max} 3399, 2919, 2850, 1690, 1652, 1624, 1542 cm^{-1}; ^1H ($CDCl_3$, 500 MHz) and ^{13}C ($CDCl_3$, 100 MHz) NMR, see Table 1; HRTOFESIMS *m*/*z* 437.2669 $[M + H]^+$ (calcd for $C_{28}H_{36}O_4$, 437.2684).

*Withasomniferolide B (**2**):* pale yellow powder; $[\alpha]^{25}_D$ −4.8 (*c* 0.06, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 220 (4.0), 350 (1.12) nm; ECD (300 μM, MeOH) λ ($\Delta\varepsilon$) 251 (+4100), 350 (−2600) nm; IR (KBr) ν_{max} 3366, 2926, 2869, 1705, 1652, 1626, 1541 cm^{-1}; ^1H ($CDCl_3$, 500 MHz) and ^{13}C ($CDCl_3$, 100 MHz) NMR, see Table 1; HRTOFESIMS *m*/*z* 437.2683 $[M + H]^+$ (calcd for $C_{28}H_{36}O_4$, 437.2684).

Synthesis of 1,5-Di-*O*-feruloylpentanediol (6). Ethyl chloroformate (1.45 mL, 15 mmol) and triethylamine (2.2 mL, 15 mmol) were added to a suspension of ferulic acid (1.47 g, 7.6 mmol) in CH_2Cl_2 (10 mL) and stirred for 1 h at −15 °C until all of of the starting material disappeared, as determined by TLC. 1,5-Pentanediol (7.6 mmol) and 4-dimethylaminopyridine (0.05 g, 0.15 mmol) were then added, and the mixture was stirred at room temperature for 6 h. The reaction mixture was then concentrated under reduced pressure to give the crude 1,5-di-*O*-(ethoxycarbonyloxy)feruloylpentanediol (**c**). This product was purified by silica gel column chromatography using *n*-hexane−ethyl acetate (7.5:2.5) as eluents to give a white solid, yield: 60%. To a solution of the protected di-*O*-feruloyl ester (**c**) (3 mmol) in CH_2Cl_2 (10 mL) was added 80 equiv of piperidine (64 mmol) at 0 °C, and the reaction mixture was stirred at room temperature for 3 h. The crude product was purified by VLC (silica gel) using *n*-hexane−ethyl acetate (1:1) as eluents to give 1,5-di-*O*-feruloylpentanediol (**6**): white, amorphous solid; yield 80%; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.61 (2H, d, *J* = 16 Hz), 7.06 (2H, dd, *J* = 1.5, 8 Hz), 7.02 (2H, d, *J* = 1.5 Hz), 6.90 (2H, d, *J* = 8.0 Hz), 6.28 (2H d, *J* = 16 Hz), 3.91 (6H, s), 4.23 (4H, t, *J* = 6.5 Hz), 1.77 (4H, m), 1.54 (2H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.3 (C-1, C-1′), 147.9 (C-6, C-6′), 146.8 (C-7, C-7′), 144.8 (C-3, C-3′), 127.0 (C-4, C-4′), 123.0 (C-9, C-9′), 115.5 (C-2, C-2′), 114.7 (C-8, C-8′), 109.3 (C-5, C-5′), 64.2 (C-1″, C-5″), 55.9 (OCH_3-3, OCH_3-3′), 28.4, (C-2″−C-4″), 22.6 (C-3″); HRTOFESIMS *m*/*z* 455.1685 $[M - H]^+$ (calcd for $C_{25}H_{27}O_8$, 455.1706).

General Procedure for the Synthesis of 4′-Ethyloxycarbonyloxy Ferulates d−f. The synthesis of 4′-ethyloxycarbonyloxy ferulates **d**−**f** was performed according to a previously described procedure.[28]

General Procedure for the Synthesis of Ferulates 7−9. The synthesis of ferulates **7**−**9** was performed according to a previously described procedure.[28]

DOI: 10.1021/acs.jnatprod.8b01023
J. Nat. Prod. XXXX, XXX, XXX−XXX

*Octadecyl ferulate (**7**):* white, amorphous solid, yield 60%; VLC mobile phase: toluene−ethyl acetate (9.5:0.5); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.61 (1H, d, J = 16 Hz, H-3), 7.07 (1H, dd, J = 2, 8 Hz, H-6′), 7.03 (1H, d, J = 2 Hz, H-2′), 6.91 (1H, d, J = 8 Hz, H-5′), 6.29 (1H, d, J = 16 Hz, H-2), 5.94 (1H, s, OH), 4.19 (2H, t, J = 6.8 Hz, H-1″), 3.92 (3H, s, OCH_3-3′), 1.69 (2H, m, H-2″), 1.40 (2H, m, H-3′′), 1.26 (28H, m, H-4′′−H-17′′), 0.88 (3H, t, J = 6.8, Hz, H-18″); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 167.3 (C-1), 147.9 (C-3′), 146.8 (C-4′), 144.6 (C-3), 127.1 (C-1′), 123.0 (C-6′), 115.7 (C-2), 114.7 (C-5′), 109.3 (C-2′), 64.6 (C-1″), 55.9 (OCH_3), 31.9 (C-16″), 29.7, 29.6, 29.5 (C-5′′−C-14′′), 29.4 (C-15′′), 29.3 (C-4′′), 28.8 (C-2′′), 26.0 (C-3′′), 22.7 (C-17′′), 14.1 (C-18′′); ESIMS m/z (negative mode) 445 $[M - H]^-$, 487 $[M + CH_3CN]^-$.

*Eicosanyl ferulate (**8**):* white, amorphous solid; yield 75%; VLC mobile phase: toluene−ethyl acetate (9.5:0.5); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.61 (1H, d, J = 16 Hz, H-3), 7.07 (1H, dd, J = 2, 8.5 Hz, H-6′), 7.03 (1H, d, J = 2 Hz, H-2′), 6.91 (1H, d, J = 8.5 Hz, H-5′), 6.29 (1H, d, J = 16 Hz, H-2), 4.19 (2H, t, J = 6.8 Hz, H-1″), 3.92 (3H, s, OCH_3-3′), 1.69 (2H, m, H-2″), 1.40 (2H, m, H-3′′), 1.26 (32H, m, H-4′′−H-19′′), 0.88 (3H, t, J = 6.5, Hz, H-20″); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 167.3 (C-1), 147.9 (C-3′), 146.8 (C-4′), 144.6 (C-3), 127.1 (C-1′), 123.0 (C-6′), 115.7 (C-2), 114.7 (C-5′), 109.3 (C-2′), 64.6 (C-1″), 56.0 (OCH_3), 31.9 (C-18″), 29.7, 29.6, 29.5 (C-5′′− C-16′′), 29.4 (C-17′′), 29.3 (C-4′′), 28.8 (C-2′′), 26.0 (C-3′′), 22.7 (C-19′′), 14.1 (C-20′′); ESIMS m/z (negative mode) 473 $[M - H]^-$, 515 $[M + CH_3CN]^-$.

*Docosanyl ferulate (**9**):* white, amorphous solid; yield 69%; VLC mobile phase: toluene−ethyl acetate (9.5:0.5); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 7.61 (1H, d, J = 16 Hz, H-3), 7.07 (1H, dd, J = 2, 8.5 Hz, H-6′), 7.03 (1H, d, J = 2 Hz, H-2′), 6.91 (1H, d, J = 8.5 Hz, H-5′), 6.29 (1H, d, J = 16 Hz, H-2), 4.19 (2H, t, J = 6.8 Hz, H-1″), 3.93 (3H, s, OCH_3-3′), 1.70 (2H, m, H-2″), 1.40 (2H, m, H-3′′), 1.26 (32H, m, H-4′′−H-21′′), 0.88 (3H, t, J = 6.5, Hz, H-22″); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 167.3 (C-1), 148 (C-3′), 146.8 (C-4′), 144.6 (C-3), 127.1 (C-1′), 123.0 (C-6′), 115.7 (C-2), 114.7 (C-5′), 109.3 (C-2′), 64.6 (C-1″), 55.9 (OCH_3), 31.9 (C-20″), 29.7, 29.6, 29.5 (C-5′′−C-18′′), 29.4 (C-19′′), 29.3 (C-4′′), 28.8 (C-2′′), 26.0 (C-3′′), 22.7 (C-21′′), 14.1 (C-22′′); ESIMS m/z (negative mode) 501 $[M - H]^-$, 543 $[M + CH_3CN]^-$.

Electrophysiology Experiments. Male Sprague−Dawley rats (Charles River, Italy), weighing 125−155 g, were maintained in controlled environmental conditions (temperature 22 ± 2 °C and humidity 60−65%), under a 12 h light/12 h dark cycle. All experiments were conducted in conformity with the regulations of the Committee for the Protection and Use of Animals of the University of Cagliari, in accordance with current Italian legislation on animal experimentation (D.L. 26/2014) and the European directives (2010/63/EU) on care and use of laboratory animals. In particular, this study was approved by the Organization for Animal Care of the University of Cagliari (OPBA-UniCA) and performed in accordance with the Ministry of Health authorization number 1177/2016-pr (December 15, 2016). Furthermore, every effort was made to minimize the number of animals used. Coronal brain slices containing the hippocampus were prepared as previously described.[33] Briefly, animals were subjected to deep anesthesia with isoflurane 2−5% and decapitated. Their brain was rapidly removed from the skull and transferred to a modified artificial cerebrospinal fluid (ACSF) containing (in mM) 220 sucrose, 2 KCl, 0.2 $CaCl_2$, 6 $MgSO_4$, 26 $NaHCO_3$, 1.3 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O_2 and 5% CO_2). Coronal brain slices (thickness, 260 μm) containing the dentate gyrus of the hippocampus were cut in ice-cold modified ACSF with the use of a Leica VT1200S vibratome (Leica, Heidelberg, Germany). Slices were then transferred immediately to a nylon net submerged in standard ACSF containing (in mM) 126 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O_2/5% CO_2) for at least 40 min at a controlled temperature of 35 °C. After subsequent incubation for at least 1 h at room temperature, hemislices were transferred to the recording chamber and continuously perfused with standard ACSF at a constant flow rate of ∼2 mL/min. All recordings were performed at room temperature.

GABAergic-evoked postsynaptic currents were recorded with an Axopatch 200-B amplifier, filtered at 2 kHz, and digitized at 5 kHz. Recording pipets were prepared from borosilicate capillaries with an internal filament using a Fleming Brown micropipet puller (Molecular Devices, Novato, CA, USA). Resistance of the pipets ranged from 4.5 to 6.0 MΩ when they were filled with, in mM, 140 CsCl, 2 MgCl, 2 CaCl, 10 EGTA, 10 HEPES, 2 ATP-Na, pH 7.3 with 5 N CsOH. Only recordings with access resistance of <25 MΩ (the values usually ranged from 9 to 20 MΩ) were analyzed. Series resistance was not compensated, and cells were excluded from a further analysis if access resistance changed by >20% during the course of the recording. A bipolar concentric stimulating electrode was placed near the granule neurons' cell bodies in order to evoke the depolarization of presynaptic terminals and thus the GABA release from nearby GABAergic interneurons. The nonselective glutamatergic receptor antagonist kynurenic acid (3 mM) was added to the solution in order to exclude excitatory currents when granule cells were clamped at −65 mV. The variation of the IPSC amplitude before and after drug perfusion was considered as the parameter for evaluating the drug effect on GABAAR activity.

Statistical Analysis. Data are presented as means ± standard error of the mean (SEM) and were compared by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test, or Student's t test with the use of Prism software (version 6, Graphpad). A p value of <0.05 was considered statistically significant.

Ligand Preparation and Qikprop Analysis. Compound **9** was prepared with Maestro GUI[34] and subject to conformational search by means of MacroModel program version 9.2.[35] Merck molecular force fields[36] were applied, and the implicit solvation model generalized Born/surface area was used.[37] Therefore, the compound geometry was energy minimized using the Polak-Ribier conjugate gradient method, 10 000 iterations, and a convergence criterion of 0.01 kcal/(mol Å). Conformational searching was performed using the MCMM method, allowing 5000 steps. The compound global minimum conformation was considered for QikProp analysis (QikProp, Schrödinger, LLC, New York, NY, USA).[38]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b01023.

Additional information (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: + 39-706758979. Fax: + 39-706758553. E-mail: cottiglf@unica.it.

ORCID

Simona Distinto: 0000-0003-1620-6225

Filippo Cottiglia: 0000-0003-4659-9808

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was financially supported by Regione Autonoma della Sardegna (RAS) (CRP26805 CUP B71J11001480002). The authors wish to thank Dr. A. Agarwal (Natural Remedies Pvt Ltd. Bangalore, India) for the generous gift of *W. somnifera* root extract.

DOI: 10.1021/acs.jnatprod.8b01023
J. Nat. Prod. XXXX, XXX, XXX−XXX

REFERENCES

(1) Weiner, M. A.; Weiner, J. In *Herbs that Heal*; Quantum Books: Mill Valley, CA, 1994; pp 70−72.
(2) Ven Murthy, M. R.; Ranjekar, P. K.; Ramassamy, C.; Deshpande, M. *Cent. Nerv. Syst. Agents Med. Chem.* **2010**, *10*, 28−246.
(3) Seenivasagam, R.; Sathiyamoorthy, S.; Hemavathi, K. *Int. J. Immunol. Stud.* **2011**, *1*, 297−317.
(4) Andrade, C.; Aswath, A.; Chaturvedi, S. K.; Srinivasa, M.; Raguram, R. *Indian J. Psychiat.* **2000**, *4*, 295−301.
(5) Bhattacharya, A.; Muruganandam, A. V.; Kumar, V.; Bhattacharya, S. K. *Indian J. Exp. Biol.* **2002**, *40*, 1161−1163.
(6) Cooley, K.; Szczurko, O.; Perri, D.; Mills, E. J.; Bernhardt, B.; Zhou, Q.; Seely, D. *PLoS One* **2009**, *4*, No. e6628.
(7) Bhattacharya, S. K.; Bhattacharya, A.; Sairam, K.; Ghosal, S. *Phytomedicine* **2000**, *7*, 463−469.
(8) Archana, R.; Namasivayam, A. *J. Ethnopharmacol.* **1998**, *64*, 91−93.
(9) Bhattacharya, S. K.; Muruganandam, A. V. *Pharmacol., Biochem. Behav.* **2003**, *75*, 547−555.
(10) Singh, B.; Chandan, B. K.; Gupta, D. K. *Phytother. Res.* **2003**, *17*, 531−536.
(11) Dhuley, J. N. *J. Ethnopharmacol.* **1998**, *60*, 173−178.
(12) Mehta, A. K.; Binkley, P.; Gandhi, S. S.; Ticku, M. K. *Indian J. Med. Res. (B)* **1991**, *9* (4), 312−315.
(13) Bhattarai, J. P.; Park, S. A.; Han, S. K. *Phytother. Res.* **2009**, *24*, 1147−1150.
(14) Orrù, A.; Marchese, G.; Casu, G.; Casu, M. A.; Kasture, S.; Cottiglia, F.; Acquas, E.; Mascia, M. P.; Anzani, N.; Ruiu, S. *Phytomedicine* **2014**, *21*, 745−752.
(15) Ruiu, S.; Longoni, R.; Spina, L.; Orrù, A.; Cottiglia, F.; Collu, M.; Kasture, S.; Acquas, E. *Behav. Pharmacol.* **2013**, *24*, 133−143.
(16) Candelario, M.; Cuellar, E.; Reyes-Ruiz, J. M.; Darabedian, N.; Zhou, F.; Miledi, R.; Russo-Neustadt, A.; Limon, A. *J. Ethnopharmacol.* **2015**, *171*, 264−272.
(17) Minguzzi, S.; Barata, L. E. S.; Shin, Y. G.; Jonas, P. F.; Chai, H.-B.; Park, E. J.; Pezzuto, J. M.; Cordell, G. A. *Phytochemistry* **2002**, *59*, 635−641.
(18) Kuroyanagi, M.; Shibata, K.; Umehara, K. *Chem. Pharm. Bull.* **1999**, *47*, 1646−1649.
(19) Pan, Y.; Wang, X.; Hu, X. *J. Nat. Prod.* **2007**, *70*, 1127−1132.
(20) Zhang, H.; Timmermann, B. N. *J. Nat. Prod.* **2016**, *79*, 732−742.
(21) Guillén-Villar, R. C.; Vargas-Álvarez, Y.; Vargas, R.; Garza, J.; Matus, M. H.; Salas-Reyes, M.; Domínguez, Z. *J. Electroanal. Chem.* **2015**, *740*, 95−104.
(22) Katagiri, Y.; Mizutani, J.; Tahara, S. *Phytochemistry* **1997**, *46*, 347−352.
(23) Menezes, J. C.; Kamat, S. P.; Cavaleiro, J. A.; Gaspar, A.; Garrido, J.; Borges, F. *Eur. J. Med. Chem.* **2011**, *46*, 773−777.
(24) Chen, L.; Liang, Y.; Song, T.; Anjum, K.; Wang, W.; Yu, S.; Huang, H.; Lian, X.-Y.; Zhang, Z. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 2629−2633.
(25) Zhang, H.; Samadi, A. K.; Gallagher, R. J.; Arya, J. J.; Tong, X.; Day, V. W.; Cohen, M. S.; Kindscher, K.; Gollapudi, R.; Timmermann, B. N. *J. Nat. Prod.* **2011**, *74*, 2532−2544.
(26) Jana, C. K.; Hoecker, J.; Woods, T. M.; Jessen, H. J.; Neuburger, M.; Gademann, K. *Angew. Chem., Int. Ed.* **2011**, *50*, 8407−8411.
(27) Neogi, P.; Kawai, M.; Butsugan, Y.; Mori, Y.; Suzuki, M. *Bull. Chem. Soc. Jpn.* **1988**, *61*, 4479−4481.
(28) Sonar, V. P.; Corona, A.; Distinto, S.; Maccioni, E.; Meleddu, R.; Fois, B.; Floris, C.; Malpure, N. V.; Alcaro, S.; Tramontano, E.; Cottiglia, F. *Eur. J. Med. Chem.* **2017**, *130*, 248−260.
(29) Bonetti, E. P.; Burkard, W. P.; Gabl, M.; Hunkeler, W.; Lorez, H.-P.; Martin, J. M.; Moehler, H.; Osterrieder, W.; Pieri, L.; Polc, P.; Richards, J. G.; Schaffner, R.; Scherschlicht, R.; Schoch, P.; Haefely, W. E. *Pharmacol., Biochem. Behav.* **1988**, *31*, 733−749.
(30) Chatterjee, S.; Srivastava, S.; Khalid, A.; Singh, N.; Sangwan, R. S.; Sidhu, O. P.; Roy, R.; Khetrapal, C. L.; Tuli, R. *Phytochemistry* **2010**, *71*, 1085−1094.
(31) Gupta, M.; Kaur, G. *NeuroMol. Med.* **2018**, *20*, 343−362.
(32) Kaur, T.; Kaur, G. *J. Neuroinflammation* **2017**, *14*, 201.
(33) Talani, G.; Biggio, F.; Licheri, V.; Locci, V.; Biggio, G.; Sanna, E. *Front. Cell. Neurosci.* **2016**, *10*, 158.
(34) *Maestro (GUI)*; Schrödinger LLC: New York, 2018.
(35) Mohamadi, F.; Richards, N. G.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440−467.
(36) Halgren, T. A. *J. Comput. Chem.* **1996**, *17*, 490−451.
(37) Kollman, P. A.; Massova, I.; Reyes, C.; Kuhn, B.; Huo, S.; Chong, L.; Lee, M.; Lee, T.; Duan, Y.; Wang, W.; Donini, O.; Cieplak, P.; Srinivasan, J.; Case, D. A.; Cheatham, T. E. *Acc. Chem. Res.* **2000**, *33*, 889−897.
(38) *Qik Prop*; Schrödinger, LLC: New York, 2018.

DOI: 10.1021/acs.jnatprod.8b01023
J. Nat. Prod. XXXX, XXX, XXX−XXX