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KHUSIMOL, A NON-PEPTIDE LIGAND FOR VASOPRESSIN V_{1a} RECEPTORS

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ABSTRACT.—In the course of a random screen of various plant extracts, khusimol [**1**], a non-peptide molecule isolated from the root of *Vetiveria zizanioides*, was found to competitively inhibit the binding of vasopressin to rat liver V_{1a} receptors (K_i=50 μM). The ^{1}H- and ^{13}C-nmr spectra of this sesquiterpene alcohol were assigned unambiguously.

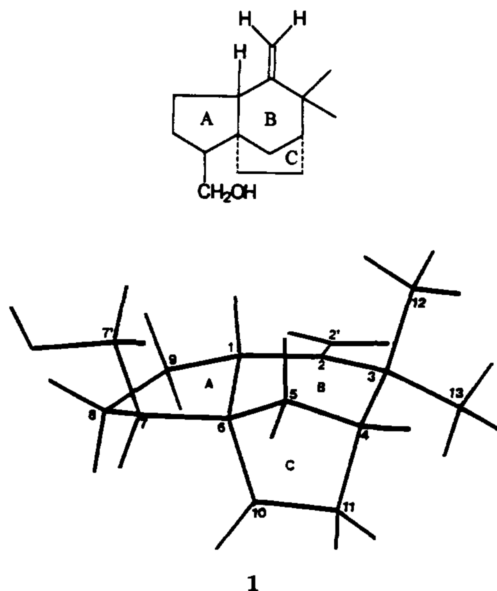
As plant materials can provide new and original chemical structures, we have engaged in a random screen of various plant extracts in the hope of isolating new active non-peptide molecules. In the course of this work, a MeOH extract of *Vetiveria zizanioides* (L.) Nash (Gramineae) roots was found to inhibit [^{3}H]-vasopressin (AVP) binding to rat liver vasopressin V_{1a} receptors with an ID_{50} value (dilution of the extract required to inhibit half the maximal specific binding) of about 1/100. Bioassay-directed fractionation of this extract led to the isolation of an active sesquiterpene alcohol, which was tentatively identified as khusimol [**1**].

Umarani *et al.* (1,2) were the first to isolate the primary alcohol khusimol [**1**] from a high boiling-point fraction of vetiver oil, and later studies (3–5) showed that **1** is actually the major component (15%) of this oil.

In order to establish whether the active compound we isolated was actually khusimol [**1**], its obtained spectral data were compared with literature values. The ir spectrum presented characteristic bands fully compatible with the previously reported ir data (1,2,6,7). Several authors have reported ^{1}H-nmr data obtained either at 60 MHz (1, 2, 6–8) or at 100 MHz (5, 7–10). In the present study, data obtained at 500 MHz were in agreement with those reported earlier. However, no previous ^{13}C-nmr data for this major component of vetiver oil was found in the literature. The unambiguous assignment of the ^{1}H- and ^{13}C-nmr spectra is reported here for the first time, along with the characterization of the action of khusimol [**1**] at rat liver AVP V_{1a} receptors.

RESULTS AND DISCUSSION

The isolated active compound [**1**] inhibited specific binding of [^{3}H]-AVP to rat liver membranes in a concentration-dependent manner (Figure 1) with an IC_{50} value of 125±30 μM and a Hill coefficient (n_H) value close to unity, compatible with a single-site competitive model. From these competition experiments an apparent inhibition constant, K_i=41±10 μM, was calculated according to the Cheng and Prusoff equation (11). Scatchard analysis of the data obtained from saturation binding experiments carried out in the absence or in the presence of the isolated active molecule (25 and 50 μM) showed that khusimol [**1**] inhibits [^{3}H]-AVP binding in a competitive manner (Figure 2; Table 1). Indeed, the apparent dissociation constant (K_d) decreased as a function of



khusimol [1] concentration, whereas the maximal binding capacity (B_{max}) was not significantly altered, giving a K_i value of 53±9 μM, in good agreement with that previously obtained in competition studies.

To date, only a few synthetic compounds have been shown to exhibit an affinity for AVP V_{1a} receptors. Recently, the AVP V_{1a}-antagonist properties of two synthetic, non-peptide, orally effective compounds belonging to different chemical series have been reported, namely, OPC-21268 and SR 49059 (12,13). These molecules display selective affinity for AVP V_{1a} receptors (K_i=0.350 μM and 0.002 μM, respectively). Because a key role of AVP has been clearly shown in several types of human cardiovascular pathology, non-peptide, orally effective AVP V_{1a} molecules appear to be promising therapeutic agents in this area. The lower but sufficient affinity of the natural product khusimol [1] is thus clearly of high interest in this field.

Comparison of the normal broad-band proton decoupled and DEPT-135 ^{13}C- nmr

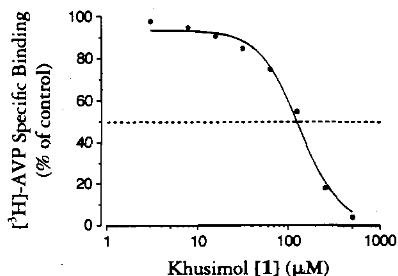


FIGURE 1. Inhibition of [^{3}H]-AVP specific binding by increasing concentrations of khusimol [1] in rat liver plasma membranes.

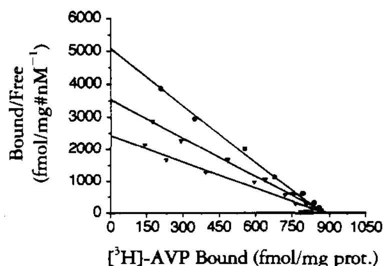


FIGURE 2. Scatchard plots of the data of a typical experiment of [^{3}H]-AVP binding to rat liver plasma membranes performed in the absence (●), or presence of 25 μM (▽) or 50 μM (▼) khusimol [1] (the experiment was performed in duplicate and repeated three times, without observing noticeable modifications).

TABLE 1. Saturation Analysis from Experiments with [^{3}H]-AVP Conducted in the Absence (Control) or Presence of 25 or 50 μM Khusimol [**1**] on Rat Liver Plasma Membranes.[a]

	Control	Khusimol [**1**] (μM)	
		25	50
K_d (nM)[b]	0.20±0.08	(0.30***±0.07)[d]	(0.42***±0.12)[d]
B_{max} (fmol/mg prot.)[c]	921±81	927±77	930±73

[a]Data are means±SD of three determinations.
[b]K_d is the apparent equilibrium dissociation constant.
[c]B_{max} is the maximal binding capacity.
[d]Significant differences between control and treated experiments are denoted by *** (p<0.001).

spectra proved the presence of three quaternary carbons, two methyl, seven methylene, and three methine groups in the molecule **1**. The integration of the 1D ^{1}H-nmr spectrum confirmed the presence of the expected number of hydrogens. Furthermore, the examination of the spectrum showed the presence of two tertiary methyl groups at 1.05 ppm (3H, s) and 1.07 ppm (3H, s), a CH_2OH methylene group having two non-equivalent protons at 3.47 ppm (1H, dd, J=7.7 and 10.3 Hz) and at 3.73 ppm (1H, dd, J=6.3 and 10.3 Hz), and a CH_2=C vinylic methylene group at 4.60 ppm (1H, t, J=1.5 to 1.8 Hz) and at 4.73 ppm (1H, t, J=1.5 to 1.8 Hz). The addition of one drop of D_2O to an aliquot of the solution established the presence of an exchangeable proton (OH).

The results of the COSY (^{1}H,^{1}H) and COSY (^{1}H,^{13}C) nmr experiments on **1**, either for direct or long-range coupling, are listed in Tables 2 and 3. The relative stereochemistry of the methyl groups of khusimol [**1**] has been discussed earlier (10). Thus, the ^{1}H-nmr signal at 1.05 ppm (correlated with the carbon signal at 26.0 ppm) was assigned to the axial methyl group, whereas the ^{1}H-nmr signal at 1.07 ppm (correlated with the carbon signal at 28.5 ppm) was assigned to the equatorial methyl group. The signals of

TABLE 2. ^{1}H-Nmr Spectral Data and (^{1}H,^{1}H) Observed Correlations for Khusimol [**1**].

Proton	Shift (δ, ppm)[a]	Multiplicity (J, Hz)	COSY[b] (observed correlations)
1	2.43	ddd (6.5, 1.8, 0.7, 11.3)	H-2'a, H-2'b, H-5b, H-9a, H-9b, H-10b
2'a	4.73	dd (1.7, 1.8)	H-1, H-2'b, H-13
2'b	4.60	dd (1.7, 0.7)	H-1, H-2'a, H-4, H-12
4	1.80	t (5.4)	H-2'b, H-10a, H-11a, H-11b, H-13
5a	1.47	m	H-5b, H-10a, H-10b, H-11a, H-11b, H-12
5b	0.98	m	H-1, H-5a, H-9a, H-10a, H-11a, H-11b
7	1.92	m	H-7'a, H-7'b, H-8a, H-8b
7'a	3.73	dd (10.4, 6.3)	H-7, H-7'b, H-8a, H-8b
7'b	3.48	dd (10.4, 7.6)	H-7, H-7'a, H-8a, H-8b
OH	1.60 to 1.32	br	
8a	1.89	m	H-7, H-7'a, H-7'b, H-8b, H-9a, H-9b
8b	1.42	m	H-7, H-7'a, H-7'b, H-8a, H-9a, H-9b
9a	1.68	dddd (6.5, 7.3, 14.8, 2.0)	H-1, H-5b, H-8a, H-8b, H-9b
9b	1.51	m	H-1, H-8a, H-8b, H-9a
10a	1.86	m	H-4, H-5a, H-5b, H-10b, H-11a, H-11b
10b	1.54	m	H-1, H-5a, H-10a, H-11a, H-11b
11a	1.46	m	H-4, H-5a, H-5b, H-10a, H-10b, H-11b, H-12
11b	1.34	m	H-4, H-5a, H-5b, H-10a, H-10b, H-11a, H-12
12	1.07	s	H-2'b, H-11a, H-11b, H-13
13	1.05	s	H-2'a, H-4, H-11a, H-11b, H-12

[a]Chemical shifts at high frequency from internal TMS.
[b](^{1}H,^{1}H) correlation nmr spectroscopy.

TABLE 3. ^{13}C-Nmr Spectral Data, Direct and Long-range Observed (^{1}H,^{13}C) Correlations for Khusimol [1].

Carbon	δ/ppm	Direct correlations	Long-range correlations
1	48.7 d	H-1 (2.43)	H-2′a, H-2′b
2	156.5 s		H-12, H-13
2′	105.3 t	H-2′a (4.73), H-2′b (4.60)	
3	40.3 s		H-12, H-13, H-2′a, H-2′b, H-5a, H-11a
4	49.3 d	H-4 (1.80)	H-5a, (H-5b)[a], H-11a, H-12, H-13
5	33.3 t	H-5a (1.47), H-5b (0.98)	H-7, H-10a
6	53.2 s		H-4, H-9b
7	48.2 d	H-7 (1.92)	H-5b, H-7′b
7′	66.4 t	H-7′a (3.73), H-7′b (3.47)	H-8b
8	26.5 t	H-8a (1.89), H-8b (1.42)	H-7, H-7′b, H-9b
9	25.1 t	H-9a (1.68), H-9b (1.51)	H-2′b, H-5b
10	35.8 t	H-10a (1.86), H-10b (1.54)	H-5a, H-11a, H-11b
11	25.4 t	H-11a (1.46), H-11b (1.34)	H-5b, H-7, H-10a
12	28.5 q	H-12 (1.07)	H-13
13	26.0 q	H-13 (1.05)	H-12

[a]Very weak correlation.

the two vinylic protons at 4.73 and 4.60 ppm showed a triplet-like multiplicity. H-2′a exhibited cross-peaks with H-2′b (geminal coupling), H-1 (allylic coupling), and H-13, whereas H-2′b was correlated with H-2′a and H-1 but had a weak correlation with both H-4 and H-12. These observations are in full agreement with earlier results on **1** (8, 10) obtained by double-irradiation techniques from which it could be inferred that the angle between the allyl hydrogen and the plane of the exocyclic double bond is nearly 110°. Besides, the high-frequency triplet could be unambiguously assigned to the proton directed towards the C-3 *gem*-dimethyl group by means of differential nOe experiments (14) (Table 4) since irradiation of either of the tertiary methyl group signals resulted in an increase in the intensity of the resonance at δ 4.73 ppm, whereas the intensity of the signal at 4.60 ppm was not modified.

In addition to H-2′a and H-2′b, H-1 of **1** may be expected to be correlated to H-9a and H-9b. Although the intensity of the COSY response is generally not simply related to the size of J, these are strong correlations due to vicinal couplings, which allowed the assignments of H-9a and H-9b at 1.68 and 1.51 ppm, respectively. H-1 exhibited a further correlation with a proton at 0.98 ppm. The signals at 3.73 and 3.47 ppm were easily assigned to the two nonequivalent protons of the CH_2OH group, H-7′a and H-7′b. In the COSY spectrum these protons were both clearly connected to two other resonances, coupled to each other, which must be the geminal pair at C-8 (δ_{H8a} 1.89 ppm, δ_{H8b} 1.42 ppm). H-8a and H-8b were further correlated to H-7 (δ 1.92 ppm), H-9a, and H-9b, thus confirming the corresponding assignments. H-9a exhibited correlations with H-9b (geminal coupling), H-7, H-8a, H-8b, and a resonance at 0.98 ppm, which we may ascribe to one of the geminal protons at C-5, namely, H-5b. In addition to the connections already mentioned with H-1 and H-9a, H-5b exhibited a strong correlation with H-5a (geminal coupling). Further correlations were observed for H-4, H-10a, H-10b, H-11a, and H-11b. As expected, H-5a showed a strong correlation with H-5b, as well as having additional connectivities with H-4, H-10a, H-10b, H-11a, and H-11b, whereas H-4 was further connected with H-5a, H-5b, H-11a, H-11b, and H-13. In addition to the connections already mentioned, H-12 was connected to H-13 and vice versa.

Further examination of the results reported in Table 4 showed unambiguously that H-5a, H-8a, H-9a, and CH_3-12 (δ 1.07 ppm) are in the β- orientation, while CH_3-13 (δ 1.05 ppm) is in the α- orientation. These experiments helped also to determine more

TABLE 4. Results of Differential NOe Experiments on Khusimol [**1**].

Irradiation	Signals enhanced (ppm)		Enhancement[a]
H-1	4.60	H-2′b	vw
	3.73	H-7′a	m
	3.48	H-7′b	m
	1.89	H-8a	m
	1.68	H-9a	w
	1.47	H-5a	w
	1.07	H-12	m
H-12			
	4.73	H-2′a	m
H-13			

[a]The enhancements are reported as s (strong), m (medium), w (weak) or vw (very weak) to prevent over-interpretation, because the data have not been corrected for partial saturation (14).

accurately the chemical shifts of these signals, which are situated in a crowded region of the spectrum of khusimol [**1**].

From the ^{1}H-nmr assignments, the ^{13}C-nmr chemical shifts of the protonated carbon atoms of **1** could be easily correlated by the one-bond (C-H) heteronuclear COSY nmr spectrum. The assignment of C-2 (δ 156.5 ppm) was straightforward and the long-range C-H correlations helped in assigning the two other quaternary carbons, C-3 and C-6, as well as confirming the assignment of C-2. In addition, the observed correlation between C-3 and H-5a and the lack of a similar correlation with H-5b clearly showed that H-5a must be the equatorial proton in the α- orientation as the dihedral angle C-3, C-4, C-5, H-5a is close to 180°, giving rise to nearly the maximal value of the 3-bond coupling constant. The results of the nOe experiments described above confirmed this assignment, inasmuch as the signal of H-5β (H-5b) was enhanced by irradiation of that of H-1.

Previous results obtained on the stereochemistry of zizanoic acid support a trans-fused A/B ring and a β-orientation for the carboxylic group (15). The biosynthesis of khusimol [**1**] (16) and the absolute configuration (2,15) have also been reported. Our results are in excellent agreement with the earlier data. Molecular modeling performed with SYBYL (Tripos Associates) followed by geometry optimization of the molecule carried out using the MM2 77 Program modified by Sanofi Recherche, fully confirmed the nmr data. The absolute stereostructure of khusimol [**1**] may thus be depicted as shown.

In conclusion, the results presented herein demonstrate for the first time the affinity of khusimol [**1**] for AVP V_{1a} receptors. Complete and unambiguous assignments of both the ^{1}H- and ^{13}C-nmr spectra of this compound were obtained using 1D and 2D nmr methods, while the results of molecular modeling helped to confirm the absolute stereostructure of the molecule. Khusimol [**1**] is a rigid natural structure which could be used as a model to develop more effective synthetic analogues of high interest in the vasopressin therapeutic field (17). For this purpose, the selectivity and the agonist or antagonist profile of khusimol [**1**] need to be further investigated in relevant biological tests.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The nmr experiments were performed at 24° on a Bruker AMX 500 spectrometer operating at 500.13 MHz for ^{1}H and at 125.77 MHz for ^{13}C, locked to the deuterium signal of the solvent and equipped with a dual ^{1}H/^{13}C 5-mm diameter probe. Data processing was performed on a Bruker ASPECT X32 data station. The concentration of the solution studied was 19.0 mg/ml in $CDCl_3$, TMS was used as internal standard, and the parameters for 1D ^{1}H-nmr spectra were spectral

width 6000 Hz, pulse angle 30°, and interpulse delay 4.7 sec. To confirm some assignments, various nOes were measured in the ^{1}H-nmr spectrum using 1D difference nOe methods. Spectra were processed with 1 Hz line broadening to reduce subtraction artifacts. Percentage enhancements were calculated using integrals. Other parameters were: 2D COSY-90 (^{1}H,^{1}H) spectra (spectral width 6098 Hz, 512 FIDs, relaxation delay 1.5 sec, number of scans 64); 1D ^{13}C-nmr spectra (spectral width 21739 Hz, pulse angle 30°, interpulse delay 4.0 sec); DEPT-135 spectra (spectral width 31250 Hz, pulse angle 90°, acquisition time 1.0 sec, relaxation delay 2.0 sec); 2D (^{1}H,^{13}C) COSY spectra for either direct (CH) (J=140 Hz) or for long-range (J=10 Hz) couplings (spectral width 3900 Hz, 256 FIDs, acquisition time 0.047 sec, number of scans 216).

PLANT MATERIAL.—The plant material was collected initially in Réunion Island, and then cultivated in the greenhouses of Sanofi Elf Bio Recherche, Labège, France. The roots were air-dried at room temperature. The plant was identified by Dr. M. Cambornac, Yves Rocher, La Gacilly, France. A voucher specimen of the plant is maintained (reference 048) in the Botanical Garden of Yves Rocher, La Gacilly, France.

EXTRACTION AND ISOLATION.—The dried, pounded roots (500 g) were extracted four times, over 3 h each, with 6 liters of MeOH at room temperature. After filtration, the solvent was removed under reduced pressure. The active residue (46 g) was dissolved in distilled H_2O and extracted with *n*-hexane. The active extract (21.5 g) was subjected to several separations on 15% $AgNO_3$-impregnated Si gel (cc and tlc) (18) to obtain 0.4 g of an active fraction in $CHCl_3$-MeOH (98:2). The fraction was purified by prep. layer chromatography on 15% $AgNO_3$-impregnated Si gel with $CHCl_3$-CH_2Cl_2-1-propanol-EtOAc (45:45:4.5:4.5) and $CHCl_3$-CH_2Cl_2 (60:40) to give khusimol [**1**] (200 mg). The molecular formula was determined by mass spectrometry as $C_{15}H_{24}O$. The ir spectrum presented characteristic bands at 3369 and 1028 cm^{-1} (CH_2OH), at 1638 and 891 cm^{-1} ($C{=}CH_2$), at 1379 and 1363 cm^{-1} (C-[CH_3]$_2$) and is thus fully compatible with the previously reported ir data (1,2,6,7). Other physical data have been published earlier (1,2), i.e., bp, n^{24}D, $[\alpha]^{27}$D as well as the ^{1}H-nmr spectrum obtained at 60 MHz. At each step of the purification process the biological activity of each fraction was monitored using the AVP V_{1a} binding assay. Unambiguous ^{1}H- and ^{13}C-nmr data for khusimol [**1**] are shown in Tables 2 and 3, respectively.

[^{3}H]-VASOPRESSIN-BINDING ASSAY.—Rat liver plasma membranes were prepared according to the procedure of Prpcic *et al.* (19) and stored as aliquots (10 mg/ml) in liquid N_2 until used. Membrane proteins were determined by the method of Bradford (20) with bovine serum albumin as the standard. The binding assay was performed as described by Granger *et al.* (21) by incubating 100 μl membrane protein (60–100 μg) in a final volume of 0.2 ml incubation medium (pH 7.4) containing 50 mM TRIS, 1 mM EGTA, 10 mM $MgCl_2$, 1 mg bacitracin, 0.1% bovine serum albumin, and 0.5 nM [^{3}H]-AVP for 60 min at 30°. The content of the assay tubes was rapidly filtered under vacuum on Whatman GF/B glass fiber filters and washed three times with 4 ml ice-cold buffer. The filters were counted in a Beta liquid scintillation counter. Non-specific binding was determined by the addition of 1 μM of unlabeled AVP and represented 10% of total binding. Saturation experiments were performed under standard conditions using increasing concentrations of [^{3}H]-AVP (60 Ci/mmole; New England Nuclear, France) from 0.05 to 25 nM. MeOH fractions resulting from each step of the purification process were assayed at a final concentration of 10% in the binding medium and tested against corresponding 10% MeOH controls.

BINDING DATA ANALYSIS.—Data are means ± SD of three experiments performed in duplicate. The IC_{50} value is defined as the inhibitor concentration required to obtain 50% inhibition of the specific binding. The inhibition constant (K_i) was calculated from the IC_{50} value using the Cheng and Prusoff equation (11) or from saturation experiments according to the equation:

$$K_i = I/[(K'_d/K_d)-1]$$

where K_d and K'_d are the equilibrium dissociation constants measured in the absence and in the presence of the inhibitor I, respectively. Binding data from saturation and competition experiments were analyzed using a non-linear regression program (22). The B_{max} and K_d values obtained in the presence or absence of test drugs were subjected to the method of Jennrich (23) for the comparison of parameters estimated by non-linear regression.

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LITERATURE CITED

1. D.C. Umarani, K.G. Gore, and K.K. Chakravarti, *Tetrahedron Lett.*, 1255 (1966).
2. D.C. Umarani, K.G. Gore, and K.K. Chakravarti, *Perfum. Essent. Oil Rec.*, **60**, 307 (1969).
3. J. Garnero, *Parf. Cosm. Sav. France,* **1**, 569 (1971).
4. A. Cazaussus, R. Pes, N. Sellier, and J.-C. Tabet, *Chromatographia,* **25**, 865 (1988).
5. A. Homma, M. Kato, M.-D. Wu, and A. Yoshikoshi, *Tetrahedron Lett.*, 231 (1970).
6. G.K. Trivedi, K.K. Chakravarty, and S.C. Bhattacharya, *Ind. J. Chem.*, **9**, 1049 (1971).
7. G.A. Neville and I.C. Nigam, *Tetrahedron Lett.*, 837 (1969).
8. G.P. Newsoroff and S. Sternhell, *Aust. J. Chem.*, **25**, 1669 (1972).
9. S. Lemberg, R.B. Hale, and B. Sawyer, *Perfum. Flavor,* **3**, 23 (1978).
10. G.A. Neville and I.C. Nigam, *Can. J. Chem.*, **47**, 2901 (1969).
11. Y. Cheng and W. Prusoff, *Biochem. Pharmacol.*, **22**, 3099 (1973).
12. Y. Yamamura, H. Ogawa, T. Chihara, K. Kondo, T. Oganawa, S. Nakamura, T. Mori, M. Tomonaga, and Y. Yabuuchi, *Science,* **252**, 572 (1991).
13. C. Serradeil-Le Gal, J. Wagnon, C. Garcia, C. Lacour, P. Guiraudou, B. Christophe, G. Villanova, D. Nisato, J.P. Maffrand, G. Le Fur, B. Cantau, C. Barberis, M. Trueba, Y. Ala, and S. Jard, *J. Clin. Invest.*, **92**, 224 (1993).
14. D. Neuhaus and M. Williamson, "The Nuclear Overhauser Effect in Structural and Conformational Analysis," VCH Publishers, Inc., New York, 1989, pp. 357–359.
15. F. Kido, H. Uda, and A. Yoshikoshi, *Tetrahedron Lett.*, 1247 (1968).
16. A. Akhila, P.K. Sharma, and R.S. Thakur, *Fitoterapia,* **58**, 243 (1987).
17. M. Manning and W.H. Sawyer, *Trends Neurosci.*, **7**, 8 (1984).
18. E. Stahl and H. Jork, "Thin Layer Chromatography," Springer Verlag, Berlin, 1990, pp. 227–228.
19. V. Prpcic, K.C. Green, P.F. Blackmore, and J.H. Exton, *J. Biol. Chem.*, **259**, 1382 (1984).
20. M.M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
21. I. Granger, C. Serradeil-Le Gal, J.-M. Augereau, and J. Gleye, *Planta Med.*, **58**, 35 (1992).
22. P.V. Munson and D. Rodbard, *Anal. Biochem.*, **107**, 220 (1980).
23. R.L. Jennrich, *Ann. Math. Stat.*, **40**, 633 (1969).

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