Spasmolytic Constituents from *Eucalyptus camaldulensis* var. *obtusa* Leaves

Sabira Begum,*,† Farhat,† Ishrat Sultana,† Bina S. Siddiqui,† Farhana Shaheen,‡ and Anwar H. Gilani‡

H. E. J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan, and Department of Physiology and Pharmacology, The Aga Khan University Medical College, Karachi-74800, Pakistan

Received May 17, 1999

Phytochemical studies on the leaves of *Eucalyptus camaldulensis* var. *obtusa* have resulted in the isolation of a new triterpenoid camaldulin (3β-formyloxyurs-11-en-28,13β-olide) (**1**) along with ursolic acid lactone acetate (**2**), ursolic acid lactone (**3**), betulinic acid (**4**), and β-sitosterol 3-*O*-β-D-glucopyranoside (**5**). The structures were assigned on the basis of 1D and 2D NMR studies. Compounds **1**−**3** were tested for spasmolytic activity and were found to possess calcium antagonist activity.

The genus *Eucalyptus* of the family Myrtaceae comprising over 500 species is indigenous to Australia, Tasmania, and the neighboring islands. *Eucalyptus camaldulensis* var. *obtusa* Dehnk, a large tree, is cultivated in the Indo-Pakistan subcontinent. Its different parts are used in traditional medicine for the treatment of diarrhea, relaxed throats, chronic dysentery, malaria, infection of the upper respiratory tract, certain skin diseases, and as an astringent in dentistry and for cuts.[1] The oil and some flavonoids of the plant[2,3] possess antifungal and antibacterial activity. Earlier chemical studies of *E. camaldulensis* var. *obtusa* have yielded various known terpenoids and flavonoids.[2−7] Several triterpenoids have previously been reported by our group, namely eucalyptic acid, eucalyptolic acid,[8] camaldulic acid, camaldulensic acid, and camaldulenic acid,[9] along with a series of known triterpenoids.

In this paper, we report the isolation of a new pentacyclic triterpenoid, camaldulin (**1**), and four known compounds, ursolic acid lactone acetate (**2**),[10] ursolic acid lactone (**3**),[11,12] betulinic acid (**4**),[13] and β-sitosterol 3-*O*-β-D-glucopyranoside (**5**),[14,15] from the fresh and uncrushed leaves of the plant. Their structures were established through 1D and 2D NMR including COSY-45, NOESY, *J*-resolved, HMQC, and HMBC spectra. This is the first report of the isolation of **2**, **4**, and **5** from this plant. Compounds **1**−**3** exhibit spasmolytic action through blockade of calcium influx.

Results and Discussion

The HREIMS of camaldulin (**1**) showed the molecular ion peak at *m*/*z* 482.3382 corresponding to the molecular formula $C_{31}H_{46}O_4$. Its UV spectrum exhibited an absorption band at 202 nm, indicating the lack of conjugation in the molecule. The ^{1}H and ^{13}C NMR data of **1** indicated that it is an α-amyrin type of triterpenoid. Its ^{1}H NMR spectrum shows five tertiary methyl signals at δ 0.86, 0.88, 0.93, 1.04, and 1.15 and two secondary methyls at δ 0.92 (d, J = 6.3 Hz) and 0.99 (d, J = 6.1 Hz). It further shows two olefinic (1640 cm^{-1}) one-proton double doublets at δ 5.93 (J = 10.3, 1.5 Hz) and 5.53 (J = 10.3, 3.2 Hz) correlated with δ_C 133.1 (C-11) and δ_C 129.1 (C-12), respectively, in the HMQC spectrum and assigned to H-11 and H-12, respectively (Table 1). These protons showed connectivities with H-9 in the COSY-45 spectrum. The assignments were confirmed through HMBC interactions (Figure 1). It may be noted that H-12 showed a large coupling (J = 3.2 Hz) with H-9, as the allylic CH (at C-9) bond is perpendicular to the C=C plane, whereas coupling between H-9 and H-11 is small (J = 1.5 Hz) because the dihedral angle between these CH bonds is about 90° in the Dreiding model of the molecule.[16]

Table 1. ^{13}C and ^{1}H NMR Spectral Data ($CDCl_3$) of **1**[a]

position	δ_C	δ_H
1	38.0	1.87, m
2	23.5	1.74, m
3	80.7	4.61, ddd (8.7, 7.0, 1.0)
4	37.9	
5	54.9	0.84, m
6	17.6	1.58, m
7	31.2	1.56, m
8	41.8	
9	53.0	1.97, dd (3.2, 1.5)[b]
10	36.4	
11	133.1	5.93, dd (10.3, 1.5)
12	129.1	5.53, dd (10.3, 3.2)
13	89.5	
14	42.0	
15	25.6	1.72, m
		1.20, m
16	22.8	2.10, m
		1.40, m
17	45.1	
18	60.6	1.64, d (12.0)
19	38.2	1.78, m
20	40.3	0.88, m
21	30.9	1.58, m
22	31.4	1.84, m
23	27.7	0.88, s
24	16.1	0.86, s
25	19.2	0.93, s
26	19.0	1.04. s
27	16.1	1.15, s
28	179.1	
29	17.9	0.99, d (6.1)
30	18.0	0.92, d (6.3)
1′	161.0	8.10, d (1.0)

[a] Values in parentheses indicate coupling constants in Hz. [b] From 2D J resolved experiment. Assignments are based on DEPT, ^{1}H, ^{1}H COSY, HMQC, and HMBC experiments and comparison with similar compounds.[10,18]

The presence of quaternary carbon signals in the ^{13}C NMR spectrum (broad band) at δ 89.5 (C-13) and 179.1 (C-28) along with a prominent $M^+ - CO_2$ peak at *m*/*z* 438.3497 in the HREIMS indicated a lactone moiety (1755 cm^{-1}) between C-13 and C-28.[17] The peaks at *m*/*z* 189.1623, 215.1755, 202.1699, and 69.0687 in the HREIMS confirmed these assignments. A one-proton doublet at δ 8.10 (J = 1.0 Hz) correlated with δ_C 161.0 in the HMQC spectrum,

* To whom correspondence should be addressed: Tel.: Off. (92-21) 473177-8, 479001-2204. Fax: 92-21 4963373, 4963124. E-mail: sabira@khi.comsats.net.pk.
† H.E.J. Research Institute of Chemistry.
‡ The Aga Khan University Medical College.

10.1021/np9902340 CCC: $19.00
Published on Web 07/12/2000

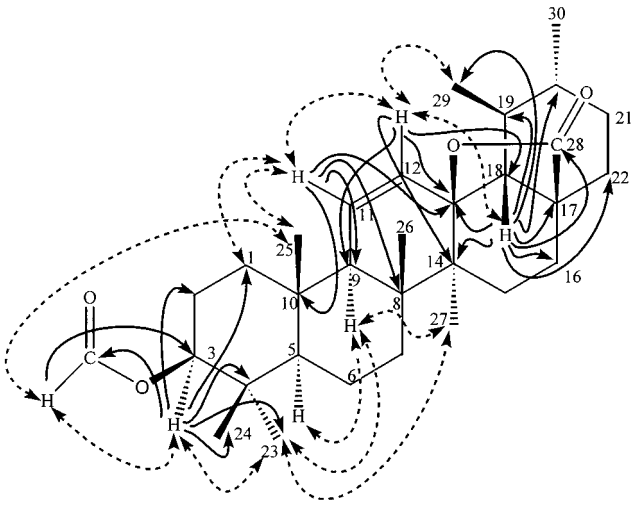

Figure 1. Significant correlations observed in HMBC (→) and NOESY (↔) spectra of **1**.

indicating a formyloxy group (2730, 1720 cm^{-1}) in the molecule,[18] which was placed at C-3 on biogenetic grounds. H-3 appeared at δ 4.61 (δ_C 80.7; HMQC) as a one-proton doublet of double doublets (J = 8.7, 7.0, and 1.0 Hz), indicating the β-orientation of the formyloxy group. A strong peak at m/z 189.1623, due to loss of formic acid from a fragment obtained through cleavage of ring C and an interaction in the COSY-45 spectrum between δ 8.10 and 4.61 supported these assignments. The assignment of the stereochemistry of **1** was achieved by 2D NOE (NOESY) NMR experiment. The major connectivities are shown in Figure 1. The HMBC data were used to connect different structural fragments as well as to confirm chemical shift (δ_H and δ_C) assignments. From the observed data, the structure of **1** was elucidated as 3β-formyloxy-urs-11-en-28,13β-olide. It may be noted that δ_C values assigned to C-11 and C-12 of **1** were comparable to those of reported values[10] for ursolic acid lactone acetate, whereas δ_H values, unambiguously assigned to these protons in the present studies on the basis of HMQC (Table 1) and HMBC (Figure 1) experiments, are the reverse of the reported values.

The known compounds (**2**−**5**) were identified by comparison of their spectral data with those of the corresponding constituents reported in the literature.[10,12−14,19−23] Our assignments of the ^{13}C and ^{1}H NMR of β-sitosterol 3-*O*-β-D-glucopyranoside agreed well with the published assignments.[15]

Compounds **1**−**3** were tested for possible spasmolytic activity via their effects on the spontaneous movements of isolated rabbit jejunum and on K^+ (50 mM)-induced contractions. All three compounds were found to be active in producing relaxation of the jejunum, and the spasmolytic activity was dose-dependent in the concentration range of 10−100 μg/mL (data not shown).

The contractions of smooth muscle preparations, including rabbit jejunum, are dependent upon an increase in the cytoplasmic free Ca^{2+}, which activates the contractile elements.[24] The increase in intracellular Ca^{2+} is due to either influx via voltage-dependent Ca^{2+} channels (VDCs) or is released from intracellular stores in sarcoplasmic reticulum. Periodic depolarization and repolarization regulate the spontaneous movements of the intestine, and, at the height of depolarization, the action potential appears as a rapid influx of Ca^{2+} via VDCs.[25] The inhibition of spontaneous movements of rabbit jejunum by the compounds tested may be due to interference either with the release process or with the Ca^{2+} influx through VDCs.

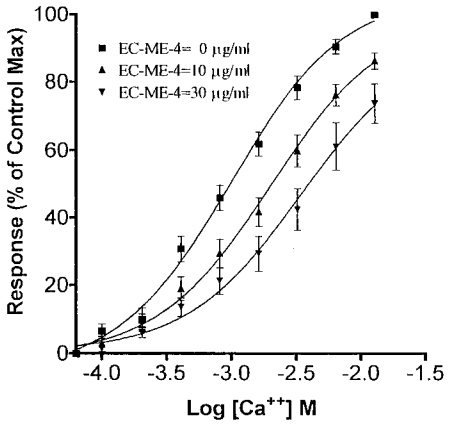

Figure 2. Dose−response curves of Ca^{2+} in the absence (control) and presence of different doses **1** in isolated rabbit jejunum. The antagonist was allowed to equilibrate with the tissue for 30 min before the re-determination of Ca^{2+} responses, and the curves represent the data point of four experiments expressed as means ± SEM. A standard calcium antagonist, verapamil, also produced qualitatively similar effect on Ca^{2+}; EC-ME−4 = camaldulin (**1**).

Table 2. Comparison of Compounds (**1**−**3**) for Their Calcium Antagonist Activity Expressed in Terms of Dose Ratios, Constructed on the Isolated Rabbit Jejunum

dose (μg/mL)	dose ratios[a]		
	1	**2**	**3**
0	1	1	1
10	2.03 ± 0.29 (4)	6.20 ± 1.37 (3)	
30	3.47 ± 0.56 (4)	17.51 ± 1.1 (5)	2.57 ± 0.73 (4)
100			7.38 ± 1.08 (4)

[a] Dose ratios represent the shifts in the Ca^{2+} dose−response curves produced by the respective dose of the antagonist. The values shown represent means ± SEM and the number of observations in parentheses beside each value.

The contractions induced by high K^+ (>30 mM) are dependent upon ingress of Ca^{2+} into the cells through VDCs,[26] and a substance that inhibits K^+-induced contractions is considered a calcium-channel blocker. Thus, inhibition of high K^+(50 mM)-induced contraction of rabbit jejunum by these compounds may be visualized as an outcome of restricted Ca^{2+} entry via VDCs. This was further confirmed when pretreatment of the tissue with the test compounds caused a dose-dependent rightward shift in the dose−response curves to Ca^{2+}, constructed in a Ca^{2+}-free medium (Figure 2). This figure shows the dose−response curves of calcium in the absence and presence of test compound **1**. The combined data of all three compounds is presented in Table 2, which shows the comparison of the shifts of calcium curves in response to different doses of the test compounds. The data indicate that compound **2** was most active as a calcium antagonist, as evidenced by its maximum displacement in the calcium curves, while the other two compounds exhibited less effect, with **1** being slightly more active.

These data indicate that compounds **1**−**3** exhibit spasmolytic action through blockade of calcium influx. The plant *E. camaldulensis* var. *obtusa* has been used in traditional medicine for the treatment of diarrhea, a hyperactive state of the gut. Calcium antagonists have been considered useful in diarrhea,[27] and it is likely that the presence of these compounds in the plant may be responsible for its folkloric use for control of diarrhea.

Experimental Section

General Experimental Procedures. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. IR and UV spectra were recorded on Jasco

A-302 and Hitachi-U-3200 spectrophotometers, respectively. Mass spectra were recorded on a Finnigan MAT 312 double-focusing mass spectrometer connected to a PDP 11/34 computer system. The ^{1}H NMR spectra were recorded on a Bruker AMX 500 NMR spectrometer operating at 500 MHz, while the ^{13}C NMR spectra were obtained on the same instrument operating at 125 MHz. The spectra were referenced to the residual solvent signals. The chemical shifts are reported in δ (ppm), and the coupling constants are in hertz. The ^{13}C NMR spectral assignments have been made partly through the comparison of chemical shifts with the published data for similar compounds[10,12,19−23] and partly through the appearance of signals in the DEPT, HMQC, and HMBC spectra. For vacuum liquid chromatography[28] (VLC), Si gel PF_{254} (E. Merck) was used, while for TLC and preparative TLC, Si gel PF_{254} (E. Merck) and precoated thin layer cards of Si gel 60 F_{254} (E. Merck) were used.

Plant Material. The leaves of the plant were collected from the Karachi region. The plant was identified by Mr. M. I. H. Brooker, Eucalypt botanist, Centre for Plant Biodiversity Research, Australian National Herbarium, Canberra, Australia, and a voucher specimen has been deposited in the herbarium.

Extraction and Isolation. Fresh, undried, and uncrushed leaves (20 kg) of *E. camaldulensis* var. *obtusa* were repeatedly extracted with EtOH at room temperature. The concentrated syrupy residue obtained on removal of the solvent under reduced pressure was partitioned between EtOAc and H_2O. The EtOAc layer was then dried (Na_2SO_4), treated with charcoal, filtered, and washed with EtOAc. The charcoal bed was further washed with MeOH−C_6H_6 (1:1). The residue left on removal of the solvent from the EtOAc filtrate and washings was divided into petroleum ether-soluble and petroleum ether-insoluble fractions. The petroleum ether-soluble fraction (123 g) was partitioned between 90% MeOH (aqueous) and petroleum ether. The residue (54 g) obtained on usual workup of 90% MeOH (aqueous) phase was subjected to VLC (petroleum ether−EtOAc, in order of increasing polarity) to afford a total of 21 pooled fractions that were obtained by combining the eluates on the basis of TLC. Fraction 4 (petroleum ether−EtOAc 9.25:0.75 eluate) on concentration and keeping overnight at room temperature gave **1** as a colorless crystalline solid, which, on crystallization from $CHCl_3$−MeOH (1:1), formed flowers of needles (70 mg). The mother liquor of **1** afforded 3β-acetoxyurs-11-en-28,13β-olide (**2**, 10 mg) by preparative TLC (petroleum ether−EtOAc, 8:2) on precoated thin layer cards of Si gel 60 F_{254} (E. Merck). Fraction 6 (petroleum ether−EtOAc, 8.75:1.25 and 8.5:1.5 eluates), on concentration and keeping overnight at room temperature, afforded betulinic acid (**3**) as a colorless crystalline solid, which crystallized as flowers of needles (1.25 g) from MeOH−C_6H_6 (2:1). Mother liquors obtained after several crystallizations (from the same solvents) were combined, concentrated, and kept cold overnight to yield crude ursolic acid lactone (**4**), which separated out as a colorless crystalline solid. On repeated crystallizations from MeOH−C_6H_6 (1:1), **4** formed colorless, fine needles (150 mg). Fraction 18 (petroleum ether−EtOAc, 2:8, 1:9; EtOAc; EtOAc−MeOH, 9.75:0.25 eluates) (6.1 g) was rechromatographed (VLC, petroleum ether−EtOAc followed by EtOAc−MeOH, in order of increasing polarity). The EtOAc−MeOH (9.95:0.05) eluate, on concentration and keeping overnight at room temperature, gave β-sitosterol-3-*O*-β-D-glucopyranoside (**5**, 140 mg) as a colorless crystalline solid, which crystallized as fine needles from $CHCl_3$−MeOH (1:1).

Camaldulin (**1**): flowers of needles ($CHCl_3$−MeOH, 1:1); mp 280−281 °C; IR ν_{max} (KBr) 2910, 2880, 2730, 1755, 1720, 1640, and 1160 cm^{-1}; UV λ_{max} (MeOH) 202 nm; HREIMS *m/z* (rel int %) 482.3382 ($C_{31}H_{46}O_4$, M^+, calcd for $C_{31}H_{46}O_4$, 482.3395) (65), 454.3461 [$C_{30}H_{46}O_3$, $M^+ - CO$] (20), 438.3497 [$C_{30}H_{46}O_2$, $M^+ - CO_2$] (100), 436.3335 [$C_{30}H_{44}O_2$, $M^+ - HCOOH$] (26), 368.2686 [$C_{25}H_{36}O_2$] (10), 300.2076 [$C_{20}H_{28}O_2$] (15), 257.1923 [$C_{18}H_{25}O$] (20), 215.1755 [$C_{16}H_{23}$] (50), 202.1699 [$C_{15}H_{22}$] (44), 189.1623 [$C_{14}H_{21}$] (36), 133.1011 [$C_{10}H_{13}$] (25), 69.0687 [C_5H_9] (77); ^{1}H and ^{13}C NMR (Table 1).

Spasmolytic Activity. Spasmolytic activitiy of the test compounds was studied by using isolated rabbit jejunum preparations, as described previously.[29] Rabbits (1.5−2.0 kg) of local Desi breed and either sex, housed at the Animal House of The Aga Khan University, Karachi, were used for this study. Segments of 2-cm length were suspended in Tyrode's solution aerated with a mixture of 95% oxygen and 5% carbon dioxide and maintained at 37 °C. The composition of the Tyrode's solution in mM was: KCl 50, NaCl 91.04, $MgCl_2$ 1.05, $NaHCO_3$ 11.87, NaH_2PO_4 0.41, $CaCl_2$ 1.8, and glucose 5.55. Intestinal responses were recorded isotonically using BioScience transducers and an oscillograph. Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug.

For the determination of calcium antagonist activity, K^+-(50 mM) was used to depolarize the preparations as described by Farre et al.[30] K^+ was added to the tissue bath, which produced a sustained contraction. The test compound was then added to the tissue bath in a cumulative fashion to obtain concentration-dependent inhibitory responses.

To confirm the calcium antagonist activity of test substances, the tissue was allowed to stabilize in normal Tyrode's solution, which was then replaced with Ca^{2+}-free Tyrode's solution containing EDTA (0.1 mM) for 30 min, to remove calcium from the tissues. This solution was further replaced with K^+-rich and Ca^{2+}-free Tyrode's solution, having the following composition (in mM): KCl 50, NaCl 91.04, $MgCl_2$ 1.05, $NaHCO_3$ 11.87, NaH_2PO_4 0.41, glucose 5.55, and EDTA 0.1. After an incubation period of 30 min, control dose−response curves of Ca^{2+} were obtained. The dose−response curves of Ca^{2+} were repeated in the presence of different concentrations of the test material, and the antagonist-induced rightward shifts (dose ratios) were used to assess the potency of the test compounds.[31,32] Dose ratios (DRs) represent the shifts in the Ca^{2+} dose−response curves produced by the respective dose of the antagonist (test material). DRs were estimated as the ED_{50} values (doses of Ca^{2+} producing 50% of the maximum response) in the presence of the respective dosage of an antagonist divided by the ED_{50} value obtained in the absence of antagonist (control curve).[32] The responses to Ca^{2+} were expressed as the percentage of the maximal response obtained in the control curve.

Supporting Information Available: Complete ^{1}H and ^{13}C NMR data of compounds **2** and **3** . This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Sastri, B. N. *The Wealth of India*; Council of Scientific and Industrial Research: New Delhi, India, 1952; Vol. 8, p 210.
(2) Chaudhari, D. C.; Suri, R. K. *Indian Perfum.* **1991**, *35*, 30.
(3) El-Gammal, A. A.; Mansour, R. M. A. *Zentralbl. Mikrobial.* **1986**, *141*, 561.
(4) Abd-alla M. F.; El-Negoumy, S. I.; El-Lakany, M. H.; Saleh, N. A. M. *Phytochemistry* **1980**, *19*, 2629.
(5) Movsumov, I. S.; Aliev, A. M. *Khim. Prir. Soedin* **1985**, 271.
(6) Dayal, R.; Maheshwari, M. L. *Indian For.* **1985**, *111*, 1077.
(7) Erazo, S.; Bustos, C.; Erazo, A. M.; Rivas, J.; Zollner, O.; Cruzat, C.; Gonzalez, J. *Plant. Med. Phytother.* **1990**, *24*, 248.
(8) Siddiqui, B. S.; Farhat; Begum, S.; Siddiqui, S. *Planta Med.* **1997**, *63*, 47.
(9) Begum, S.; Farhat; Siddiqui, B. S. *J. Nat. Prod.* **1997**, *60*, 20.
(10) Katai, M.; Terai, T.; Meguri, H. *Chem. Pharm. Bull.* **1983**, *31*, 1567.
(11) Dayal, R. *Curr. Sci.* **1987**, *56*, 670.
(12) Wang, H.; Fujimoto, Y. *Phytochemistry* **1993**, *33*, 151.
(13) Siddiqui, S.; Hafeez, F.; Begum, S.; Siddiqui, B. S. *J. Nat. Prod.* **1988**, *51*, 229.
(14) Iribarren, A. M.; Pomilio, A. B. *J. Nat. Prod.* **1983**, *46*, 752.
(15) Backhouse, N.; Delporte, C.; Negrete, R.; Suarez, S.; Cassels, B. K.; Breitmaier, E.; Schneider, C. *Int. J. Pharmacogn.* **1997**, *35*, 49.
(16) Pavia, D. L.; Lampaman, G. M.; Kriz, G. S. *Introduction to Spectroscopy*; W. B. Saunders: London, 1979; pp 116, 126.
(17) Cheung, H. T.; Wong, C. S. *Phytochemistry* **1972**, *11*, 1771.
(18) Papanov, G.; Bozov, P.; Malakov, P. *Phytochemistry* **1992**, *31*, 1424.
(19) Alam, M. S.; Chopra, N.; Ali, M.; Niwa, M. *Phytochemistry* **1996**, *41*, 1197.
(20) Kamel, M. S.; Koskinen, A. *Phytochemistry* **1995**, *40*, 1773.
(21) Jares, E. A.; Tettamanzi, M. C.; Pomilio, A. B. *Phytochemistry* **1990**, *29*, 340.
(22) Holland, H. L.; Diakow, P. R. P.; Taylor, G. J. *Can. J. Chem.* **1978**, *56*, 3121.

(23) Alam, M. S.; Chopra, N.; Ali, M.; Niwa, M.; Sakae, T. *Phytochemistry* **1994**, *37*, 521.
(24) Karaki, H.; Weiss, G. *Life Sci*. **1988**, *42*, 111.
(25) Brading, A. F. *TIPS* **1981**, *2*, 261.
(26) Bolton, T. B. *Physiol. Rev*. **1979**, *59*, 606.
(27) Brunton, L. L. In *The Pharmacological Basis of Therapeutic*, 9th ed.; Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., Gilman, A. G., Eds. McGraw-Hill: New York, 1996; Chapter 38, pp 917−936.
(28) Coll, J. C.; Bowden, B. F. *J. Nat. Prod*. **1986**, *49*, 934.
(29) Gilani, A. H.;Janbaz, K. H.; Zaman. M.; Lateef, A.; Tariq, S. R.; Ahmed, H. R. *Arch. Pharmacol. Res*. **1994**, *17*, 145.
(30) Farre, A. J.; Columbo, M.; Fort, M.; Gutierrez, B. *Gen. Pharamacol.* **1991**, *22*, 171.
(31) Gilani, A. H.; Cobbin, L. B. *Naunyn-Schmeideberg's Arch. Pharmacol.* **1986**, *332*, 16.
(32) Arunlakhshana, O.; Schild, H. O. *Br. J. Pharmacol*. **1959**, *14*, 48.

NP9902340