Maxikdiol: A Novel Dihydroxyisoprimane as an Agonist of Maxi-K Channels

Sheo B. Singh,* Michael A. Goetz, Deborah L. Zink, Anne W. Dombrowski, Jon D. Polishook, Maria L. Garcia, William Schmalhofer, Owen B. McManus and Gregory J. Kaczorowski
Merck Research Laboratories, PO Box 2000 Rahway, NJ 07065, USA

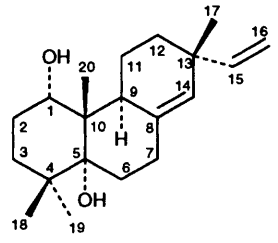
Maxi-K (large-conductance calcium-activated potassium)channels are a family of proteins which are thought to play an important role in controlling the contractility of airway smooth muscle. Maxikdiol is a novel, 1,5-dihydroxyisoprimane, diterpenoid which acts as an agonist of the channel. The isolation, structure elucidation and biological activity of maxikdiol are described.

Large-conductance, calcium-activated potassium (Maxi-K) channels are members of a large family of proteins that are prevalent in neuronal tissue and smooth muscle. These channels, which are gated by intracellular Ca^{2+} and membrane potential, allow the movement of potassium ions across the cell's plasma membrane in response to increases in internal calcium concentration and cell depolarization.[1] Activation of Maxi-K channels will promote membrane hyperpolarization which, in turn, should decrease Ca^{2+} influx through voltage-gated Ca^{2+} channels. Agonists of another type of K^+ channel present in smooth muscle—the ATP dependent K^+ channel—have been shown to relax airways smooth muscle *in vitro*, in part by this mechanism.[2] It is expected that an activator of Maxi-K channels will have potential utility in relaxing airway smooth muscle, might have anti-inflammatory activity through blockage of tachykinin release from airways neurons and might act as a bronchodilator.[3] Such agonists, therefore, could be of therapeutic benefit in asthma. Study of the physiological roles of these channels became possible only by the recent discovery of scorpion venom peptides such as charybdotoxin (ChTX) which are potent blockers of these channels.[4]

In order to discover an activator of Maxi-K channels and prove the effectiveness of this approach, natural products screening of plant and fermentation extracts was initiated using binding of [^{125}I] ChTX to Maxi-K channels[5] in bovine aortic sarcolemmal membrane vesicles. From a plant source [*Desmodium adscendens* (Sw.) DC. Var. *adscendens* of family Papillionaceae], we recently reported the discovery of a series of glycotriterpenes (soyasaponins) as potent agonists of Maxi-K channels.[6] Now we report the first agonist of the channel from our fermentation screening. The extract of the fermentation broth of an unidentified coelomycete gave a positive response in the ChTX binding screen and bio-assay guided purification resulted in the isolation and identification of the compound **1** named herein as maxikdiol, an activator of the Maxi-K channel.

A dried methyl ethyl ketone extract of the solid fermentation of this unidentified coelomycete, MF5717, was triturated with methylene dichloride and the soluble portion of the extract was chromatographed over silica gel followed by chromatography over Sephadex LH-20 and reversed phase preparative HPLC to afford maxikdiol **1** as a white powder.

Structure Elucidation.—The fast atom bombardment mass spectrum (FAB–MS) of maxikdiol **1** using dithiothriotol/dithioerythritol as matrix gave a pseudo molecular ion at *m/z* 459 (M + H + matrix) which is shifted by 6 daltons with lithium spiking (*m/z* 465: M + Li + matrix). The strongest ion in the FAB spectrum was observed *m/z* 441 and results from the neutral loss of water from the molecular ion. High resolution EI–MS indicated an empirical formula of $C_{20}H_{32}O$. Thus, from both of these techniques the molecular formula $C_{20}H_{32}O_2$ was



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derived for **1** which was consistent with the ^{13}C NMR data (Table 1). The molecular formula of maxikdiol indicated 5 degrees of unsaturation. The IR spectrum of maxikdiol showed strong hydroxyl absorption bands.

The ^{13}C NMR spectrum (DEPT and APT) of maxikdiol revealed carbons for four methyl groups, two olefinic methines, an olefinic methylene, an olefinic quaternary, six aliphatic methylenes, three aliphatic quaternary, an aliphatic methine and two oxygen-bearing carbons one being methine and the other quaternary. With the two degrees of unsaturation accounted for by the olefins, a tricyclic ring system must account for the remaining degrees of unsaturation.

The 400 MHz ^{1}H NMR spectrum of maxikdiol in CD_2Cl_2 and, in particular, C_6D_6 (Table 1) supported the ^{13}C NMR assignments. From ^{1}H NMR spectra it was obvious that all four methyl groups are angular methyls and the olefin methine had no vicinal proton. ^{1}H–^{1}H connectivities were determined by 2D ^{1}H–^{1}H COSY,[7] which besides revealing all vicinal connectivities also revealed allylic coupling of 14-H with 9-H and 7-H$_a$. TOCSY experiment in C_6D_6 (6 mmol) further extended the coupling network and revealed additional correlation of 14-H with 7-H$_e$. Examination of the Dreiding model suggests a dihedral angle of ~ 80° between 14-H with 9-H and 7-H$_a$. These protons are arranged out-of-plane and are strongly coupled. No such coupling could be seen between 14-H and 7-H$_a$ because these protons are coplanar.

The carbon spectrum (Table 1) was assigned by application of HETCOR,[8] HMQC and HMBC[9] (Table 2) experiments. The tricyclic isoprimane ring system was ascertained from HMBC experiment ($^nJ_{XH}$ = 7 Hz). Placement of the C-1 and C-5 hydroxy groups was based on the common HMBC correlations with the 20-methyl group. Additionally, as expected, C-5 was also correlated with both the 18- and 19-methyl groups as well as with 9-H. There were no correlations with 1-H due to the broadening of the proton signal. Interestingly, the 1-OH group showed a strong correlation with C-1. All the other correlations are summarized in Table 2. The isoprimane skeleton was also supported by comparison of ^{13}C NMR shifts with literature values from the elegant work of Wenkert.[10]

Table 1 NMR Assignments of maxikdiol in deuteriobenzene and deuteriodichloromethane

	CD_2Cl_2		C_6D_6		
Position	δ_C	δ_H	δ_C	δ_H (6 mmol)	δ_H (28 mmol)
1-H[e]	73.60	3.75, br s	73.40	3.40, ddd, 5.4, 5.2, 2.8	3.44, br s
1-OH				1.78, d, 5.4	1.80
2-H_e	26.50	1.60, m	26.25	1.32, dddd, 14.4, 5.2, 4.0, 2.8	1.38, m
H_a		1.95, m		1.61, m	1.68, m
3-H_e	30.50	1.98, m	30.30	1.98, m	1.82, m
H_a		1.05, m		0.87, m	0.87, m
4	39.00	—	38.77	—	—
5-OH	79.10	3.40, br s	79.00	3.18, br s	2.80, br s
6-H_e	27.60	1.40, m	27.50	1.40, m	1.27, m
H_a		1.62, m		1.40, m	1.35, m
7-H_e	30.60	2.02, m	30.40	2.00, m	1.90, m
H_a		2.46, ddddd, 14.4, 13.2, 6.2, 2.4, 1.6		2.68, m	2.32, m
8	137.20	—	136.70	—	—
9-H_a	38.30	3.07, ddddt, 8.8, 6.8, 3.2, 3.2	38.00	3.22, m	2.94, m
10	44.50	—	44.26	—	—
11-H_e	18.80	1.50, m	18.60	1.56, m	1.50, m
H_a		1.67, m		1.34, m	1.38, m
12-H_e	35.00	1.45, m	34.8	1.48, m	1.40, m
H_a		1.55, m		1.52, m	1.50, m
13	37.80	—	37.64	—	—
14	129.30	5.29, m	129.30	5.42, mt, 2.8	5.17, m
15	149.70	5.81, dd, 10.4, 17.6	149.30	5.89, dd, 10.8, 17.6	5.70, dd, 10.8, 17.6
16	110.00	4.88, dd, 10.8, 1.6	110.16	4.99, dd. 10.8, 1.6	4.78, dd, 10.8, 1.6
		4.95, dd, 17.6, 1.6		5.07, dd, 17.2, 1.6	4.84, dd, 17.6, 1.6
17	25.90	1.09, s	25.90	1.15, s	0.95, s
18	25.80	1.00, s	25.76	0.90, s	0.86, s
19	28.30	1.07, s	28.24	1.01, s	0.81, s
20	17.90	0.91, s	17.85	0.69, s	0.68, s

a Is axial and *e* is equatorial.

Table 2 HMBC correlation of maxikdiol in C_6D_6 (6 mmol) solutions

Carbon	^1H
C-1	2a-H, 20-H, 1-OH
C-2	3-H_e
C-3	2-H_a, 18-H, 19-H
C-4	18-H, 19-H
C-5	9-H, 18-H, 19-H, 20-H
C-6	7-H_a
C-7	9-H, 14-H
C-8	7-H_a, 9-H
C-9	14-H, 20-H
C-10	1-OH, 2-H, 9-H, 11-H_e, 12-H_a, 20-H
C-11	9-H
C-12	14-H, 15-H, 17-H
C-13	14-H, 15-H, 16-H, 17-H
C-14	7-H_a, 9-H, 15-H, 17-H
C-15	14-H, 16-H, 17-H
C-16	—
C-17	15-H
C-18	19-H
C-19	2-H_e, 18-H
C-20	9-H

a Is axial and *e* is equatorial.

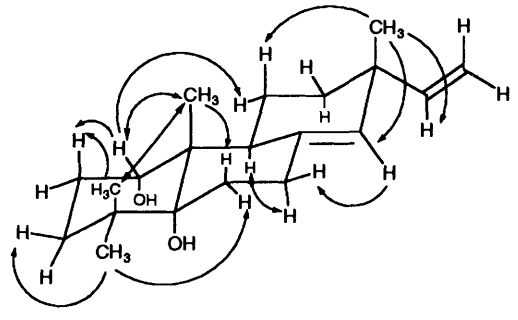

Fig. 1 Maxikdiol (arrows representing observed NOEs)

Stereochemistry.—The stereochemistry of maxikdiol was determined by NOEDS experiments in both C_6D_6 and CD_2Cl_2 as indicated in Fig. 1. The lack of diaxial coupling of 1-H with 2-H (axial) indicated that the hydroxy group is located in the axial position at C-1, this was also supported by the NOE enhancements from 1-H to 11-H_e and 20-H (methyl group). Additionally, irradiation of 20-H gave enhancements to 18-Me and 6-H_a placing them in a 1,3-diaxial arrangement in a chair conformation. This geometry was further corroborated by NOE from 19-Me to 6-H_e. Based on these results the hydroxy group at C-5 was safely placed axial (α) and the ring fusion determined as *trans*. None of these NOEs would be possible if the ring was *cis* fused and 5-OH was β. Similarly, NOEs were observed between 9-H and 7-H_a, placing them in 1,3-diaxial positions of a chair conformation. There was an NOE between 7-H_e and 14-H indicating the spatial proximity of these two protons and indicating their co-planar positioning and explaining the lack of allylic coupling. Irradiation of 17-Me gave enhancements to 15-H, 14-H and 11-H_a. This NOE supports the placement of the vinyl group at an equatorial position at C-13 and supported the isoprimane skeleton over primane. Based on a combination of data from all these NMR experiments, the structure for maxikdiol is proposed as 1α,5α-dihydroxy-Δ^8-isoprimane **1**. This represents the first example (based on CAS online substructure searching) of an isoprimane/primane skeleton

containing a 5-hydroxy group. ^{1}H NMR chemical shift differences at different concentrations of C_6D_6 solutions are probably due to different degrees of solvation of maxikdiol with the aromatic solvent and shielding. These effects were not observed in CD_2Cl_2. Therefore, complete NMR assignment became necessary in the latter solvent for future comparisons. The absolute stereochemistry of maxikdiol was not independently determined. It is based on the negative optical rotation which is common among the isoprimanes.

Biological Activity.—Maxikdiol caused a maximal inhibition of 90% of [^{125}I]ChTX binding in aortic sarcolemma, with a K_i of 1 μmol dm^{-3}. In contrast, high concentrations of maxikdiol (100 μmol dm^{-3}) had no significant effect on [^{125}I]ChTX binding to voltage-dependent K^+ channels in rat brain synaptic plasma membranes,[11] indicating specificity in the interaction of this compound with Maxi-K channels. The partial inhibition of [^{125}I]ChTX binding in smooth muscle membranes is a preliminary indication that maxikdiol is an allosteric modulator of toxin binding in this system. This postulate was confirmed by two experimental approaches; in a Scatchard analysis, maxikdiol produced a concentration-dependent decrease in the affinity of [^{125}I]ChTX with no effect on the B_{max} of this ligand in the range 1–5 μmol dm^{-3}; 10 μmol dm^{-3} maxikdiol markedly increased the dissociation rate of ChTX from its receptor suggesting that it is not a competitive inhibitor of ChTX binding. In order to determine whether maxikdiol has functional effects on Maxi-K channels, channel activity was monitored in excised inside-out patches of membrane from bovine aortic smooth muscle cells[12] in the absence or presence of test compound. Maxikdiol increased the activity of Maxi-K channels (threshold effect at 1 μmol dm^{-3}, clear increases in channel activity at 3–10 μmol dm^{-3}) when added to the cytoplasmic surface of the channel. The increase in Maxi-K activity was reversible as wash-out of maxikdiol returned channel activity to baseline levels. The biological profile of maxikdiol is very similar to that of structurally dissimilar Maxi-K channel agonist glycotriterpene;[5,6] both agents may interact at the same site on the cytoplasmic surface of the Maxi-K channel.

Experimental

General Procedure.—All the reagents and deuteriated solvents were obtained from Aldrich Chemical Company and were used without any purification. NZ amine Type A was from Sheffield Products. Yeast extract and malt extract were from Difco. Junlon was obtained from the Kouyok Trading Company (Tokyo). All other materials were reagent grade. E. Merck (Darmstadt) and/or Analtech silica gel plates (0.25 mm) were used for TLC and developed either with 3% ceric sulfate in 1.5 mol dm^{-3} H_2SO_4 spray and/or iodine vapours. Stationary phases used for column chromatography were E. Merck silica gel (70–230 or 60–63 mesh). Melting points were uncorrected.

Spectral Measurements.—The IR absorption spectra were obtained with a multiple internal reflectance cell (MIR, ZnSe on neat 10–20 μg samples. Mass spectra were recorded on JEOL SX-102A (electron impact, EI, 90 eV and fast atom bombardment, FAB). Exact mass measurements were performed at high resolution (HR–EI) using perfluorokerosene (PFK) as internal standard. Trimethylsilyl derivatives were prepared with a 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide (BSTFA)–pyridine at room temperature. The FAB spectrum was run in a matrix of dithiothreitol/dithioerthritol (20:80).

^{1}H NMR chemical shifts in CD_2Cl_2 and C_6D_6 are given relative to the solvent peaks at δ 5.32 and 7.15, respectively. ^{13}C NMR chemical shifts in CD_2Cl_2 and C_6D_6 are given relative to the solvent peak at δ 53.8 and 128.5.

^{1}H–^{1}H-COSY spectra were recorded using the standard pulse sequence of Bax *et al.*[7] The 2K–2K data set was accumulated in 1024 increments with 24 transients, respectively, for each value of t_1 for full-phase cycling. The TOCSY experiment was acquired on Varian Unity 400 MHz spectrometer, standard pulse sequence with 71 ms mixing time was used. ^{1}H–^{13}C Chemical shift correlation spectra (COSY:HETCOR) were recorded using standard pulse sequence of Bax and Morris.[8] The 1K × 4K data set was accumulated in 64 increments with 704 transients for each value of t_1. The delay time between transients was 1.0 s and the experiment was optimized for $^1J_{CH}$ = 140 Hz. The corresponding long-range experiment was optimized for a multiple bond carbon–proton coupling constant of 7 Hz. Inverse mode HMQC and HMBC experiments were performed using pulse sequence of Bax *et al.*[9] The 1K × 4K data set was recorded for HMQC experiment at Unity 400 MHz spectrometer employing Bird nulling of 0.300 s, number of increments = 512, 8 transients per increments, 0.9 s relaxation delay per transient and J_{CH} = 140 MHz. An HMBC experiment was recorded using the similar experiment with 32 transients per increments, 2.5 s of relaxation delay, and $^nJ_{XH}$ optimized for 7 Hz. NOEs were measured using NOE difference programs in CD_2Cl_2 and C_6D_6 and the sample was not degassed. In NOE difference method (Bruker 250 MHz), a relaxation delay of 2.0 s, irradiation time of 0.75–1.0 s and decoupler power of 40 L was used.

Fermentation of Maxikdiol.—The unidentified coelomycete (MF5717, deposited in the Merck Culture Collection, was isolated from the bark of *Carpinus caroliniana* in West Virginia.[13] It was preserved in YMEJ seed in 10–15% glycerol and kept as frozen vegetative mycelia (FVM) at −75 °C until use. YMEJ consisted of the following in g dm^{-3} distilled water: yeast extract 4.0, malt extract 8.0, glucose 4.0, Junlon 1.5 (pH 7.0, dispensed at 54 cm^3/250 cm^3 unbaffled Erlenmeyer flask, with cotton closures, and autoclaved 121 °C for 20 min).

Seed. An FVM was used to start a seed of the culture in YMEJ (0.5–1.0 cm^3 inoculum). The flasks were incubated at 25 °C, with agitation (220 rpm). After 4 days, the culture was heavily grown and pelleted. To break up the pellets and provide a more homogeneous inoculum to the production vessel, the seed growth was shaken with several small sterile porcelain balls and cylinders, for 1–2 h. Even with this treatment, the growth was still somewhat pelleted and difficult to pipette.

Production. An aliquot (18–24 cm^3) of the seed was used to inoculate each production vessel. The seed was added to the liquid portion of the production medium, shaken and poured into a 4 dm^3 roller bottle containing 1250 cm^3 of sterile vermiculate. The bottle was shaken vigorously to coat the vermiculate. The production vessels were then incubated on a roller machine for 21 days at 25 °C (4 rpm). [The liquid portion of the production medium consisted of the following in g dm^{-3}: glucose 150.0, urea 4.0, NZ amine type A 4.0, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.25, KCl 0.25, $ZnSO_4 \cdot 7H_2O$ 0.9 and $CaCO_3$ 16.5 (no pH adjustment). It was dispensed at 425 cm^3 1 dm^{-3} Erlenmeyer flask. Cotton closures were used, and the medium was sterilized at 121 °C for 15 min.]

Isolation of Maxikdiol.—The solid-state production fermentation (4 dm^3) broth was extracted with ethyl methyl ketone (4 dm^3) by shaking the flasks for 30–60 min, after which the filtered extract was concentrated to dryness. The residue was taken up in methylene dichloride (100 cm^3), filtered and fractionated on a 150 cm^3 silica gel column eluted with CH_2Cl_2–EtOAc (19:1 followed by 9:1). The partially purified oily

residue thus obtained was triturated with a little methanol and the insoluble material filtered off. The dried filtrate was chromatographed on Sephadex LH-20 (2 dm^3) in methanol, which afforded the compound at 0.75–0.8 column volumes of eluate. Final purification of the active fraction was achieved on a Whatman Partisil-10 ODS 3 (9.4 × 250 mm) HPLC column eluting at 4 cm^3 min^{-1} with 55% MeCN and 45% water. Fractions eluting between 29–32 min were freeze dried to afford pure maxikdiol (9.5 mg) as an amorphous powder (crystallization efforts were unsuccessful and sample generally oiled out). A benzene solution of maxikdiol was lyophilized to give a colourless solid which melted at 113–115 °C. The homogeneity of maxikdiol **1** was verified in several TLC systems and by HPLC (Whatman Partisil-5 ODS 3, operated at 40 °C, 70% aqueous MeCN at 1 cm^3 min^{-1}; $k' = 3.7$); $[\alpha]_D^{25}$ −6.6 (*c* 0.15, MeOH); ν_{max}(thin film ZnSe)/cm^{-1} 3359, 2945, 1455, 1217, 1046, 995, 915 and 857; for NMR see Table 1; *m/z* (FAB–MS) 459 (M + H + matrix), 441 (M − H_2O + H + matrix), 287 (M − H_2O + H) and 269 (M + H − $2H_2O$); lithium spike spectrum 471 (M + 2Li + matrix − H), 465 (M + H + Li + matrix) and 311 (M + Li); *m/z* (HREI–MS) 286.2315 (M − H_2O. Calc. for $C_{20}H_{30}O$: 286.2300.

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