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<p>(21) International Application Number: PCT/US94/14491</p> <p>(22) International Filing Date: 9 December 1994 (09.12.94)</p> <p>(30) Priority Data: 166,499 13 December 1993 (13.12.93) US</p> <p>(60) Parent Application or Grant (63) Related by Continuation US 166,499 (CON) Filed on 13 December 1993 (13.12.93)</p> <p>(71) Applicant (for all designated States except US): MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07067 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): GARCIA, Maria, L. [ES/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). GIACOBBE, Robert, A. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). HENSENS, Otto, D. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). KACZOROWSKI, Gregory, J. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). LEE, Seok, H. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065</p>	<p>(US). McMANUS, Owen, B. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). ZINK, Deborah, L. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).</p> <p>(74) Common Representative: MERCK & CO., INC.; Patent Dept., 126 East Lincoln Avenue, Rahway, NJ 07065 (US).</p> <p>(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KR, KZ, LK, LR, LT, LV, MD, MG, MN, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, US, UZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: NOVEL POTASSIUM CHANNEL AGONIST PRODUCED FROM TRICHODERMA VIRENS</p>		
<p>(57) Abstract</p>		
<p>The present invention relates to novel potassium channel agonists which are useful in activating the calcium activated Maxi-K potassium channel in mammalian neuronal and smooth muscle tissue. The claimed compounds are of general formula (II). In addition, a novel microbiological process for producing the claimed compounds is described.</p>	<p style="text-align: right;">(II)</p>	

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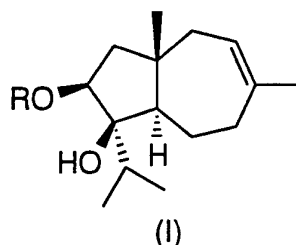
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Novel Potassium Channel Agonist Produced from *Trichoderma virens*

BACKGROUND OF THE INVENTION

5 This invention relates to novel potassium channel agonists useful to treat diseases or disorders associated with potassium channels. The invention also relates to chemical intermediates useful in the synthesis of potassium channel agonists.

10 The art reveals that a compound of structural formula (I):



wherein R is hydrogen or an acyl group has been isolated from the fungal organism *Gliocladium virens* IFO9166. Watanabe *et al. Journal of Natural Products* 53:5:1176-1181 (1990), and was disclosed as useful as a possible antifungal or antimicrobial agent. The compound wherein R is hydrogen is known in the art as CAF-603 and is hereinafter referred to as Compound B, and was referred to by Watanabe as CAF-603. The present invention, on the other hand, presents novel compounds useful as a potassium channel agonists.

25 Potassium channel agonists are useful for a number of physiological disorders in mammals, including humans. Ion channels, including potassium channels, are found in all mammalian cells and are involved in the modulation of various physiological processes and normal cellular ionic homeostasis. Potassium ions generally control the resting potential of cell membranes and the efflux of potassium ions causes repolarization of the plasma membrane after cell depolarization. Potassium channel agonists hyperpolarize the cell and diminish electrical excitability. There are a number of different potassium channel subtypes and one of the most important physiologically is the high conductance Ca²⁺-activated K⁺ (Maxi-K) channel which is present in neuronal tissue

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and smooth muscle. Intracellular calcium concentration (Ca^{2+}_i) and membrane potential gate these channels. For example, Maxi-K channels are opened by increases in intracellular calcium ion concentration or membrane depolarization. Elevation of intracellular calcium
5 concentration is required for neurotransmitter release and for smooth muscle contraction. Therefore, modulation of Maxi-K channel activity affects transmitter release from the nerve terminal and the contractability of various smooth muscle tissues. The compounds of this invention are therefore useful in the treatment of neurological disorders in which
10 hyperpolarization of neuronal cells elicits anticonvulsive and antiischemic effects. They are also useful to hyperpolarize and relax smooth muscles including, but not exclusively, those in the vasculature and airways to elicit antihypertensive and antiasthmatic actions, respectively. As smooth muscle relaxants, potassium channel agonists
15 are useful in a variety of pathophysiological conditions that include: (1) relieving muscle cramps associated with dysmenorrhea; (2) treatment of urinary incontinence; (3) treatment of irritable bowel syndrome; and (4) treatment of angina.

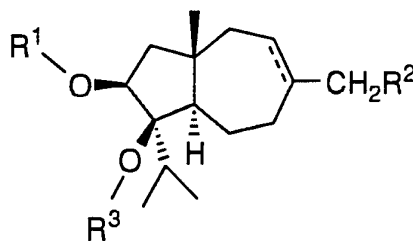
A number of drugs in development function as potassium
20 channel agonists. Examples of these include the structural classes represented by the cromakalim and pinacidil type potassium channel openers. These potassium channel agonists exert their effects primarily by opening ATP-dependent K^+ channels. They have demonstrated vasodilatory activity due to their activation of these channels in vascular
25 smooth muscle cells. Furthermore, they also activate ATP-dependent K^+ channels in tracheal smooth muscle suggesting a possible utility as antiasthmatic drugs. These compounds have either weak effects or no effects on Maxi-K channels. The present invention represents the first sesquiterpene which is useful as a potassium channel agonist. The
30 present invention relates to novel carotane sesquiterpenes which are useful as potassium channel agonists. Because these compounds target the Maxi-K channel, they have utility in suppressing neurotransmitter release, as well as in relaxing vascular and airways smooth muscle.

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SUMMARY OF THE INVENTION

The present invention is directed to novel sesquiterpenes of structural formula (II), shown below.

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(II)

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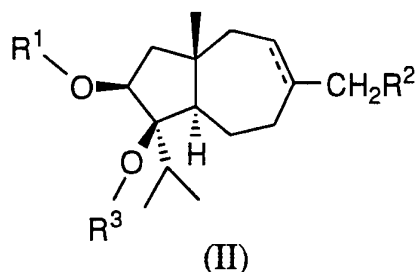
These compounds are useful as potassium channel agonists. More specifically, these compounds and pharmaceutically acceptable salts thereof are useful as Maxi-K potassium channel agonists and are thus useful in the treatment of neuronal disorders in which neurotransmitter release must be suppressed in order to elicit anticonvulsant and antiischemic activity. This compound and related compounds are also useful as smooth muscle relaxants for antiasthmatic, antihypertensive and antispasmodic applications. Furthermore, this compound is useful for other disorders sensitive to potassium channel activating activity. In addition, compounds disclosed and claimed in the instant disclosure are useful as synthetic intermediates in the synthesis and/or manipulation of the claimed potassium channel agonists. The present invention is also directed to pharmaceutical compositions containing the claimed potassium channel agonists as the active ingredient and to methods of treatment of human or mammalian diseases using the claimed compounds or pharmaceutical compositions.

DETAILED DESCRIPTION OF THE INVENTION

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The present invention is directed to compounds of formula (II):

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(II)

and pharmaceutically acceptable salts thereof, wherein:

10 R¹ is:

- (a) H,
- (b) C₁-5alkyl,
- (c) C₂-5alkenyl,
- (d) C₁-30alkylcarbonyl,
- (e) C₂-30alkenylcarbonyl,
- (f) R⁴-O-R⁴-,
- (g) substituted R⁴-O-R⁴- wherein the substituent is phenyl or methoxy,
- (h) tetrahydropyran, or
- (i) (R⁴)₃ Si-R⁴- wherein R⁴ is independently C₁-5alkyl or C₂-5 alkenyl at each occurrence,

15

20

R³ is:

- (a) H,
- (b) C₁-5alkyl,
- (c) C₂-5 alkenyl,
- (d) C₁-30alkylcarbonyl,
- (e) C₂-30alkenylcarbonyl,
- (f) R⁴-O-R⁴-,
- (g) substituted R⁴-O-R⁴- wherein the substituent is phenyl or methoxy,
- (h) tetrahydropyran, or
- (i) (R⁴)₃ SiR⁴,

25

30

or R¹ and R³ together with the adjacent oxygen and ring carbon atoms, form a five-membered ring wherein R¹ and R³ together are a single

- 5 -

substituted or unsubstituted carbon or boron atom, wherein the carbon atom is substituted with two substituents independently selected from:

- (a) H,
- (b) C₁₋₄ alkyl,
- 5 (c) C₂₋₅ alkenyl,
- (d) C₁₋₆ alkoxy,
- (e) C₂₋₆ alkenyloxy, and
- (f) phenyl, and

wherein the boron atom is substituted with one substituent selected from:

- 10 (a) H,
- (b) C₁₋₄ alkyl,
- (c) C₂₋₅ alkenyl,
- (d) C₁₋₆ alkoxy,
- (e) C₂₋₆ alkenyloxy, and
- 15 (f) phenyl;

R² is:

- (a) H,
- (b) OH,
- (c) C₁₋₅alkyloxy,
- 20 (d) C₂₋₅alkenyloxy,
- (e) C₁₋₃₀alkylcarbonyloxy,
- (f) C₂₋₃₀alkenylcarbonyloxy,
- (g) R⁴-O-R⁴-O-,
- (h) substituted R⁴-O-R⁴-O- wherein the substituent is phenyl or
- 25 methoxy,
- (i) tetrahydropyran-oxy, or
- (j) (R⁴)₃ Si-R⁴-,

provided that when R² is H and R³ is H, R¹ is not H or CH₃CO; R⁴ is independently C₁₋₅alkyl or C₂₋₅ alkenyl at each occurrence, and

30 the dashed line indicates the optional presence of a double bond.

Preferred are compounds wherein:

R¹ is:

- (a) H,
- (b) CH₃,

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- 5 (c) C₁-30alkylcarbonyl,
 (d) C₂-30alkenylcarbonyl,
 (e) CH₃OCH₂,
 (f) PhCH₂OCH₂,
 (g) t-BuOCH₂,
 (h) CH₃OCH₂CH₂OCH₂,
 (i) tetrahydropyran, or
 (j) Me₃SiCH₂CH₂;

R² is:

- 10 (a) H,
 (b) OH,
 (c) methoxy,
 (d) C₁-30alkylcarbonyloxy,
 (e) C₂-30alkenylcarbonyloxy,
 15 (f) CH₃OCH₂O,
 (g) PhCH₂OCH₂O,
 (h) t-BuOCH₂O,
 (i) CH₃OCH₂CH₂OCH₂O,
 (j) tetrahydropyranoxy, or
 20 (k) Me₃SiCH₂CH₂O,

provided that when R² is H and R³ is H, R¹ is not H or CH₃CO;
 and

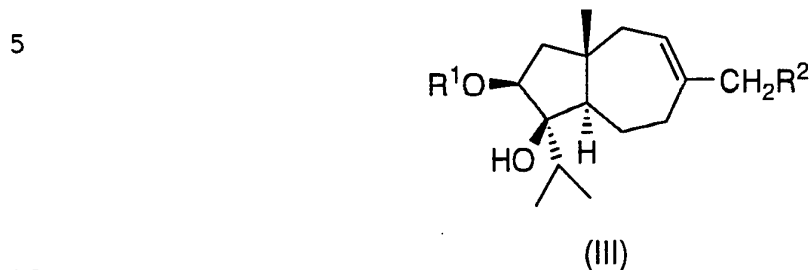
R³ is:

- 25 (a) H,
 (b) C₁-5alkyl,
 (c) C₂-5 alkenyl,
 (d) C₁-30alkylcarbonyl,
 (e) C₂-30alkylcarbonyl,
 (f) R⁴-O-R⁴-,
 30 (e) substituted R⁴-O-R⁴- wherein the substituent is phenyl or
 methoxy,
 (f) tetrahydropyran, or
 (g) (R⁴)₃ SiR⁴, and

R⁴ is independently C₁-5alkyl at each occurrence.

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The claimed invention is also directed to a compound
formula (III):



wherein:

R¹ is:

- 15
- (a) H,
 - (b) CH₃,
 - (c) C₁-30alkylcarbonyl,
 - (d) C₂-30alkenylcarbonyl,
 - (e) CH₃OCH₂,
 - (f) PhCH₂OCH₂,
 - (g) t-BuOCH₂,
 - 20 (h) CH₃OCH₂CH₂OCH₂,

R² is:

- 25
- (a) H,
 - (b) OH,
 - (c) methoxy,
 - (d) C₁-30alkylcarbonyloxy,
 - (e) C₂-30alkenylcarbonyloxy,
 - (f) CH₃OCH₂O,
 - (g) PhCH₂OCH₂O,
 - (h) t-BuOCH₂O,
 - 30 (i) CH₃OCH₂CH₂OCH₂O,

provided that when R² is H, R¹ is not H or CH₃CO.

Especially preferred potassium channel agonists are of
structural formula (III) wherein:

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R¹ is:

- (a) H,
- (b) CH₃,
- (c) C₁-30alkylcarbonyl,
- 5 (d) C₂-30alkenylcarbonyl,
- (e) CH₃OCH₂,
- (f) PhCH₂OCH₂,
- (g) t-BuOCH₂,
- (h) CH₃OCH₂CH₂OCH₂,

10 R² is:

- (a) H,
- (b) methoxy,
- (c) C₁-30alkylcarbonyloxy,
- (d) C₂-30alkenylcarbonyloxy,
- 15 (e) CH₃OCH₂O,
- (f) PhCH₂OCH₂O,
- (g) t-BuOCH₂O,
- (h) CH₃OCH₂CH₂OCH₂O,

provided that when R² is H, R¹ is not H or CH₃CO.

20

The compounds of the present invention may have asymmetric centers and occur as racemates, racemic mixtures and as individual enantiomers or diastereomers, with all isomeric forms and mixtures thereof being included within the scope of the present invention.

25

When any variable (e.g., aryl, alkyl, R¹, R², etc.) occurs more than one time in any constituent or in a structural formula, its definition on each occurrence is independent of its definition at every other occurrence. Also, combinations of substituents and/or variables are permissible only if such combinations result in stable compounds.

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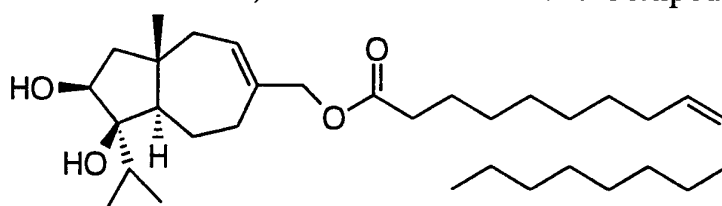
Also included within the scope of this invention are pharmaceutically acceptable salts or esters, where a basic or acidic group is present in a compound of formula II, such as, for example on the substituted alkyl moiety. When an acidic substituent is present, i.e. -COOH, there can be formed the ammonium, sodium, calcium salt, and

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the like, for use as the dosage form. Also, in the case of the -COOH group being present, pharmaceutically acceptable esters may be employed, e.g. acetate, maleate, pivaloyloxymethyl, and the like, and those esters known in the art for modifying solubility or hydrolysis characteristics for use as sustained release or prodrug formulations. Where a basic group is present, such as amino, acidic salts such as hydrochloride, hydrobromide, acetate, pamoate and the like may be used as the dosage form.

As used herein "alkyl" is intended to include both branched- and straight-chain saturated aliphatic hydrocarbon groups having the specified number of carbon atoms and includes methyl (Me), ethyl (Et), propyl (Pr), butyl (Bu), pentyl, hexyl and the like. "Alkoxy" represents an alkyl group of the indicated number of carbon atoms attached through an oxygen bridge. "Cycloalkyl" is intended to include saturated carbon ring groups such as cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl (Cyh). "Alkenyl" is intended to include hydrocarbon groups of either a straight or branched configuration with one or more carbon-carbon double bonds which may occur in any stable point along the chain, such as ethenyl, propenyl, butenyl, and the like. "Halo" or "halogen" as used herein means fluoro, chloro, bromo and iodo.

In addition, the invention relates to Compound A:

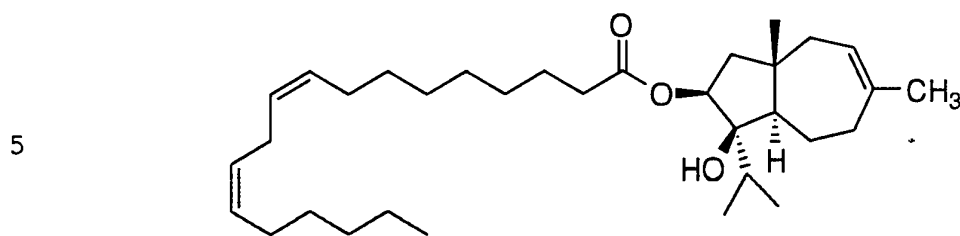


Compound A

The invention is also directed to a compound selected from the group consisting of:

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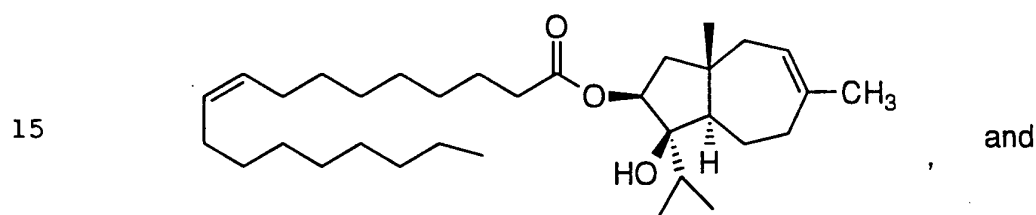
(a)



Compound C

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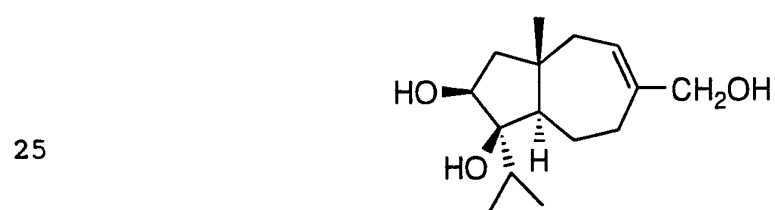
(b)



Compound D

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(c)



Compound E

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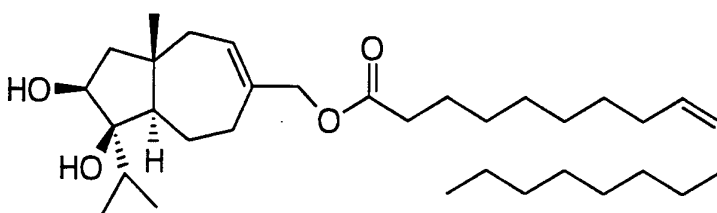
The primary production process used to produce the compounds claimed in the instant invention is through the use of the well known fungal organism *Trichoderma virens* (ATCC 74180). As described in more detail *infra*, Compound A may be produced in laboratory or large scale quantities utilizing *T. virens* under suitable

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conditions. This compound may then be purified to a substantially pure form after sufficient and desirable quantities of the potassium channel agonist are produced by *T. virens*. The novel natural product potassium channel agonist isolated from the fungal broth of *T. virens* has the formula:

5

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Compound A

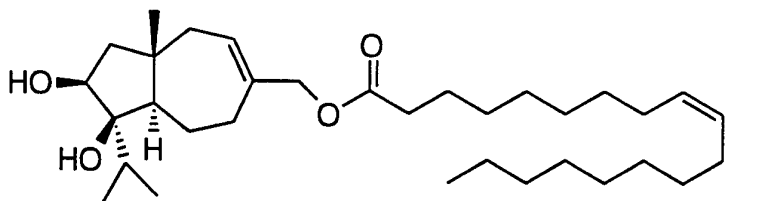
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The method of producing the compounds of the present invention comprises:

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(a) culturing a fungal organism *Trichoderma virens* (ATCC 74180) so that the fungal organism produces Compound A:

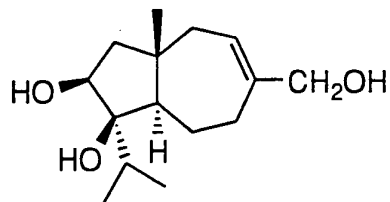
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(b) purifying the Compound A; and
 (c) optionally reacting the purified product of step (b) with dilute sodium hydroxide to produce a Compound E of the formula:

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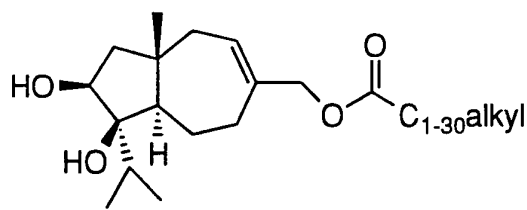


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Compound E

which is further reacted after suitable protection of the vicinal hydroxyl group with a compound selected from a C₁₋₃₀ alkyl or alkenyl anhydride or a C₁₋₃₀ alkyl or alkenyl halide and deprotected to produce a compound with potassium channel agonist activity of the formula:

10



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(IV)

Biological Production of Compound A:

20

The culture Merck Culture Number MF5783 is that of a fungus isolated from sheep dung, near Aborlí, Tarragona, Spain which has been identified as *Trichoderma virens* (Miller, Giddens & Foster) von Arx (Hyphomycetes). This culture exhibits all the essential morphological characteristics of that species (Domsch *et al.*, 1980; Bissett, 1991). In the literature, this fungus is commonly referred to by its synonym *Gliocladium virens* Miller, Giddens & Foster. This culture has been deposited with the American Type Culture Collection at 12301 Parklawn Drive, Rockville, MD 20852 as ATCC 74180 under conditions of the Budapest Treaty.

30

The culture MF5783, identified as *Trichoderma virens* exhibits the following morphological features:

Colonies growing rapidly on oatmeal agar (Difco Laboratories), 25°C, 12 hr photoperiod, after 4 days attaining 75-78 mm in diameter, appressed to slightly raised, with downy to radially floccose

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aerial mycelium, developing scattered, moist conidial pustules in older portions of colonies, dull, with an even, appressed margin, hyaline at margin but soon white, pale yellowish green, Pale Olive Buff, Yellowish Glaucous, to dull green, Corydalis Green, Dark Greenish Glaucous with
5 development of conidial pustules (capitalized color names from Ridgway, 1912). Reverse similar in color. Odors and exudates absent.

Colonies growing moderately fast on Emerson Yp Ss (Difco Laboratories) agar, 25°C, 12 hr photoperiod, after 4 days attaining 40-43 mm diameter, appressed toward margin, slightly raised toward center,
10 obscurely radially striate, also obscurely zonate, with velvety to irregularly floccose aerial mycelium, with margin irregular to minutely fimbriate, appressed, with minute droplets of moist conidia in older regions, mixed with irregular pale greenish gray conidial pustules, hyaline at margin, but soon yellowish green to green, Yellowish
15 Glaucous, Water Green, Deep Lichen Green, Deep Greenish Glaucous, Grape Green, Leaf Green. Reverse similar in color. Odors and exudates absent.

Colonies growing very rapidly on corn meal agar (Difco Laboratories), 25°C, 12 hr photoperiod, after 4 days attaining 80 to >90
20 mm in diameter, with appressed to radially floccose aerial mycelium, translucent to pale grayish green, with dull green conidial pustules. Reverse translucent. Odors and exudates absent. No growth occurred at 37°C on on Emerson Yp Ss agar after 7 days.

Conidial pustules forming in irregular, semi-confluent zones,
25 with individual pustules up to 2 mm wide, without sterile appendage protruding from the surface, often with moist conidial droplets on the surface. Conidiophores up to 200 µm tall, 2-6 µm wide, indeterminate or determinate, often terminating in a verticillate whorl of conidiogenous cells, unbranched or with 1-4 simple branches, branches dichotomous or
30 verticillate, septate, occasionally with fine incrustations on the walls near the base. Conidiogenous cells, enteroblastic, phialidic, 5-14 x 3-5 µm, solitary or in verticils of 2-5, either arising directly from main conidiophore axis, lageniform to ampulliform, symmetrical or eccentric, narrowed at conidiogenous locus, with conidiogenous locus developing a

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flared collarete in age. Conidia broadly ellipsoid, to obovate or pyriform, sometimes slightly curved, often tapered at proximal end, 5-9 x 3-5 μm , smooth, thin-walled, hyaline to pale green in KOH, adhering together in moist droplets at apex of conidiogenous cells.

5 Chlamydospores abundant on vegetative hyphae after a few days, terminal or intercalary, globose, subglobose to pyriform, 4.5-9 μm in diameter, smooth, thick-walled, with refractive contents. Hyphae septate, branched, sometime incrustated. References which were helpful in the identification of the above microorganism include Bissett, J. (1991). A
10 revision of the genus *Trichoderma*. II. Infrageneric classification. *Canadian Journal of Botany* 69:2357-2372; Domsch, K.H., W. Gams and T. Anderson. (1980). *Compendium of Soil Fungi*. Academic Press, London; Hammill, T. M. (1970). *Paecilomyces clavisporeis* sp. nov., *Trichoderma saturnisporum* sp. nov., and other noteworthy soil fungi
15 from Georgia. *Mycologia* 62:107-122; and Ridgway, R. (1912). *Color Standards and Nomenclature*. Published by the author, Washington, D.C.

The above fungal organism may be used in the production of Compound A which is a potassium channel agonist.

In general the Compounds of structural formulae (I) and (II),
20 particularly Compounds A and B, may be produced by culturing (fermenting) the above described strain, Merck number MF5783 (ATCC 74180), in a solid brown rice-based fermentation medium containing assimilable carbon and nitrogen sources preferably under static aerobic conditions, under constant fluorescent light until a substantial amount of
25 compounds of Compounds A and B is detected in the fermentation extract. The culture is incubated in a solid brown rice-based medium at a temperature between 20°C and 37°C, preferably 25°C for a period of time necessary to complete the formation of Compounds A and B, usually for a period between 3 to 28 days, preferably between 14 to 21
30 days. In addition, the above described strain can be incubated under submerged aerobic conditions with shaking, preferably on a rotary shaker operating at 220 rpm with a 5 cm throw, with all other incubation parameters remaining the same as for the solid-based medium. The aqueous production medium is maintained at a pH between 5 and 8,

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preferably about 6.0, at the initiation and termination (harvest) of the fermentation process. The desired pH may be maintained by the use of a buffer such as [2-(N-morpholino) ethanesulfonic acid] monohydrate (MES), 3-(N-morpholino)propane sulfonic acid (MOPS), phosphate
5 buffer and the like, or by choice of nutrient materials which inherently possess buffering properties, such as production media described herein below. The active compound is extracted from the mycelial growth of the culture with an alcoholic or oxygenated solvent, such as an ester or ketone. The preferred solvent for extraction is methyl ethyl ketone
10 (MEK). The solution containing the desired compound is concentrated *in vacuo* and subjected to chromatographic separation to isolate Compounds A and B from the cultivation medium.

The preferred sources of carbon in the nutrient medium include mannitol, glucose, sucrose, glycerol, xylose, galactose, fructose,
15 lactose, sorbitol, starch, dextrin, other sugars and sugar alcohols, starches and other carbohydrates, or carbohydrates derivatives, and the like. Other sources which may be included are maltose, rhamnose, raffinose, arabinose, mannose, salicin, sodium succinate, acetate, and the like as well as complex nutrients such as brown rice, millet, oat flour, yellow
20 corn meal, rice, cracked corn, and the like. The exact quantity of the carbon source which is utilized in the medium will depend, in part, upon the other ingredients in the medium, but it is usually found that an amount of carbohydrate between 0.5 and 15 percent by weight of the medium is satisfactory. These carbon sources can be used individually or
25 several such carbon sources may be combined in the same medium.

The preferred sources of nitrogen are yeast extract, NZ Amine type E, yellow corn meal, meat extract, peptone, gluten meal, cottonseed meal, soybean meal and other vegetable meals (partially or totally defatted), casein hydrolysates, soybean hydrolysates and yeast
30 hydrolysates, corn steep liquor, dried yeast, wheat germ, feather meal, peanut powder, distiller's solubles, etc., as well as inorganic and organic nitrogen compounds such as ammonium salts (e.g. ammonium nitrate, ammonium sulfate, ammonium phosphate, etc.), urea, amino acids such as serine, alanine, proline, glycine, arginine or threonine, and the like.

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The various sources of nitrogen can be used alone or in combination in amounts ranging from 0.2 to 10 percent by weight of the medium.

The carbon and nitrogen sources, though advantageously employed in combination, need not be used in their pure form because
5 less pure materials which contain traces of growth factors and considerable quantities of mineral nutrients are also suitable for use. When desired, there may be added to the medium inorganic salts, sodium, potassium, magnesium, calcium, phosphate, sulfate, chloride, carbonate, and like ions which can be incorporated in the culture medium as sodium
10 or calcium carbonate, sodium or potassium phosphate, sodium or potassium chloride, sodium or potassium iodide, magnesium salts, copper salts, cobalt salts, and the like. Also included are trace metals such as cobalt, manganese, iron, molybdenum, zinc, cadmium, copper, and the like. The various sources of inorganic salts can be used alone or in
15 combination in amounts ranging from 0.1 to 1.0 , and trace elements ranging from 0.001 to 0.1 percent by weight of the medium.

If necessary, especially when the culture medium foams seriously, a defoaming agent, such as polypropylene glycol 2000 (PPG-2000), liquid paraffin, fatty oil, plant oil, mineral oil or silicone may be
20 added.

Conditions for static solid based fermentation conditions for the production of Compounds A and B are detailed below. In addition, production of these compounds also occurs under submerged aerobic conditions by shaking the culture, preferably at 220 rpm on a rotary
25 shaker with a 5 cm throw. Furthermore, when the growth is carried out in large tanks, it is preferable to use the vegetative form of the organism for inoculation in the production tanks in order to avoid growth lag in the process of production of Compounds A and B. Accordingly, it is desirable first to produce a vegetative inoculum of the organism by
30 inoculating a relatively small quantity of culture medium with spores or mycelia of the organism produced in a "slant" or from previously prepared frozen mycelium and culturing said inoculated medium, also called the "seed medium", and then to transfer the cultured vegetative inoculum aseptically to large tanks. The seed medium, in which the

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inoculum is produced may be seen in Table 1 and is generally autoclaved to sterilize the medium prior to inoculation. The seed medium is generally adjusted to a pH between 5 and 8, preferably about 6.8, prior to the autoclaving step by suitable addition of an acid or base, preferably as a dilute solution such as hydrochloric acid or sodium hydroxide. Growth of the culture in this seed medium is maintained between 20°C and 37°C, preferably 25°C. Incubation of culture MF5783 (ATCC 74180) in seed medium (Table 1) is usually conducted for a period of about 2 to 6 days, preferably 3 to 4 days, on a rotary shaker operating at 220 rpm with a 5 cm throw; the length of incubation time may be varied according to fermentation conditions and scales. If appropriate, a second stage seed fermentation may be carried out in the seed medium (Table 1) for greater production of mycelial mass by inoculating fresh seed medium with a portion of the culture growth and then incubating under similar conditions but for a shortened period. The resulting growth then may be employed to inoculate the production medium 1, 2 or 3 (Table 2, 3 and 4). The fermentation production medium 1 inoculated with the seed culture growth is incubated for 3 to 28 days usually 14 to 21 days with agitation at 25°C. Agitation and aeration of the culture mixture may be accomplished in a variety of ways. Agitation may be provided by a propeller or similar mechanical agitation equipment, by revolving or shaking the fermentor, by various pumping equipment or by the passage of sterile air through the medium. Aeration may be effected by passing sterile air through the fermentation mixture.

Preferred seed and production media for carrying out the fermentation include the following media as described in Tables 1-4.

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Table 1

<u>SEED MEDIUM</u>				
		<u>Trace Element Mix</u>		
	<u>per liter</u>		<u>per liter</u>	
5	Corn Steep Liquor	5 g	FeSO ₄ •H ₂ O	1 g
	Tomatoe Paste	40 g	MnSO ₄ •4H ₂ O	1 g
	Oat Flour	10 g	CuCl ₂ •2H ₂ O	25 mg
	Glucose	10 g	CaCl ₂	100 mg
10	Trace element mix	10 mL	H ₃ BO ₃	56 mg
			(NH ₄) ₆ Mo ₇ O ₂₄ •4H ₂ O	19 mg
			ZnSO ₄ •7H ₂ O	200 mg
	pH = 6.8			

15

Table 2Production Medium 1

	<u>Component</u>	<u>Amount</u> (per 250-mL flask)
20	Brown Rice	10.0 g
	Yeast Extract	20.0 mg
	Sodium Tartrate	10.0 mg
	KH ₂ PO ₄	10.0 mg
25	Distilled Water	20.0 mL

pH was not adjusted prior to autoclaving for 20 min.
Immediately before use, the medium was moistened
with 15 mL of water and autoclaved again for 20 min.

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Table 3
Production Medium 2

	<u>per liter</u>
D-Mannitol	100 g
NZ-Amine (type E)	33 g
FIDCO-Yeast Extract	10 g
Ammonium Sulfate	5 g
Potassium Phosphate (monobasic)	9 g
no pH adjustment	

Table 4
Production Medium 3

Component	Amount (per 250-ml flask)
Millet	15.0 g
Ardamine PH	0.5 g
Sodium Tartrate	100.0 mg
FeSO ₄ •7H ₂ O	10.0 mg
Monosodium Glutamate	100.0 mg
Corn Oil	0.1 mL
Distilled Water	15.0 mL

pH was not adjusted prior to autoclaving for 20 min.
Immediately before use, the medium was moistened
with 15 ml of water and autoclaved again for 20 min.

Compounds C, D and E are useful as synthetic intermediates
in the synthesis of the claimed potassium channel agonists. For example,
the vicinal hydroxyl moieties may be protected with a variety of
protecting groups including alkyl esters such as those depicted above.
The allylic methyl group may then be oxidized to the allylic alcohol
which can then be derivatized to form the claimed active esters at R².

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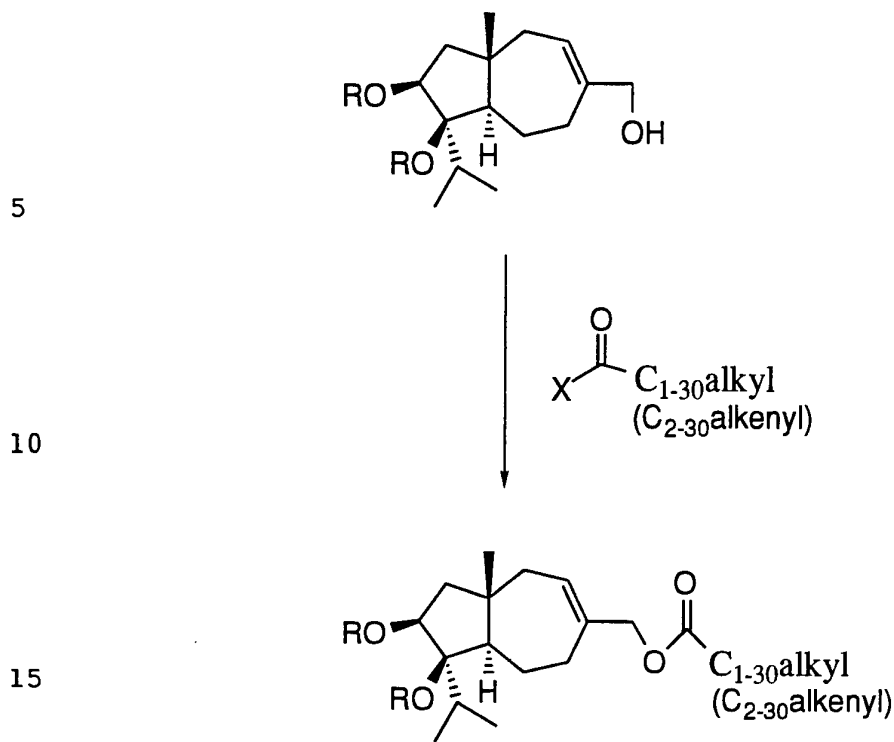
The use of the compounds above provides an alternative synthetic route to the compounds of the instant invention.

In addition, Compound A isolated in substantially purified form from the microorganism *Trichoderma virens* (ATCC 74180) can be hydrolyzed to Compound E or derivatives thereof wherein the vicinal hydroxyl moieties are protected with common protecting groups. Compound E and hydroxyl protected derivatives thereof can, in turn, be used in the nonbiological synthetic production of C₁₋₃₀ alkyl esters, or C₂₋₃₀ alkenyl esters which are also useful as potassium channel agonists. Scheme 1 provides a general description of this process. The allylic hydroxyl compound is reacted under suitable basic conditions (to generate an anion) with the activated carbonyl compound of the general formula (XCOC₁₋₃₀alkyl or XCOC₂₋₃₀alkenyl wherein X is a conventional leaving group such as a halogen) to form ester which is deprotected and subsequently used as a potassium channel agonist. The alkyl esters may be selected from, for example, the methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, octadecyl series of up to thirty carbon atoms and including both saturated and unsaturated systems. The preferred ester is the oleate ester.

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SCHEME 1

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In addition, compounds of formula (II) wherein R^1 and R^3 together are a substituted or unsubstituted carbon or boron atom forming a 5-membered ring, wherein the carbon atom may be disubstituted and the boron atom may be monosubstituted may be produced by adding an excess of substituted carboxylic acid or substituted boric acid in ether to an ethereal solution of a compound of structural formula (II) wherein R^1 and R^3 are both hydroxy. The reaction mixture may be monitored and the product may be recovered by preparatory thin layer chromatography.

The invention is also directed to a method of activating a potassium channel in a mammal, including a human, comprising administering a nontoxic pharmacologically effective amount a potassium channel agonist of the present invention. The potassium channel agonist of the present invention may be administered orally or parenterally in the form of a capsule, a tablet, an injectable preparation or the like. It is usually desirable to use the oral route. Doses may be

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varied, depending on the age, severity, body weight and other conditions of human patients, but daily dosage for adults is within a range of from about 1 mg to 2000 mg (preferably 5 mg to 200 mg) which may be given in two to four divided doses. Higher doses may be favorably employed
5 as required.

The invention also relates to a method of maintaining or inducing hyperpolarization of a mammalian cell containing a potassium channel comprising the administration to a mammal, including a human, of a nontoxic pharmacologically effective amount of a potassium channel
10 agonist as described in the present invention. The potassium channel agonist of the present invention may be administered orally or parenterally in the form of a capsule, a tablet, an injectable preparation or the like. It is usually desirable to use the oral route. Doses may be varied, depending on the age, severity, body weight and other conditions
15 of human patients, but daily dosage for adults is within a range of from about 1 mg to 2000 mg (preferably 5 mg to 200 mg) which may be given in two to four divided doses. Higher doses may be favorably employed as required.

Furthermore, the invention relates to a method of treating
20 neuronal disorders such as convulsions or ischemia comprising administering to a patient in need of treatment thereof a nontoxic pharmaceutically effective amount of the potassium channel antagonists of the present invention or their pharmaceutically acceptable salts or hydrates. The potassium channel agonist of the present invention may be
25 administered orally or parenterally in the form of a capsule, a tablet, an injectable preparation or the like. It is usually desirable to use the oral route. Doses may be varied, depending on the age, severity, body weight and other conditions of human patients, but daily dosage for adults is within a range of from about 1 mg to 2000 mg (preferably 5 mg to 200
30 mg) which may be given in two to four divided doses. Higher doses may be favorably employed as required.

The invention further relates to a method of treating smooth muscle disorders such as such as hypertension, asthma, dysmenorrhea, urinary incontinence, and irritable bowel syndrome comprising

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administering to a patient in need of treatment thereof a nontoxic pharmaceutically effective amount of the potassium channel antagonists of the present invention or their pharmaceutically acceptable salts or hydrates. The potassium channel agonist of the present invention may be administered orally or parenterally in the form of a capsule, a tablet, an injectable preparation or the like. It is usually desirable to use the oral route. Doses may be varied, depending on the age, severity, body weight and other conditions of human patients, but daily dosage for adults is within a range of from about 1 mg to 2000 mg (preferably 5 mg to 200 mg) which may be given in two to four divided doses. Higher doses may be favorably employed as required.

Pharmaceutical compositions comprising a compound of the present invention or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier may be formed. The pharmaceutically acceptable salts of the compounds of this invention include those formed from cations such as sodium, potassium, aluminum, calcium, lithium, magnesium, zinc and from bases such as ammonia, ethylenediamine, N-methyl-glutamine, lysine, arginine, ornithine, choline, N,N'-dibenzylethylenediamine, chlorprocaine, diethanolamine, procaine, N-benzylphenethylamine, diethylamine, piperazine, tris(hydroxymethyl)aminomethane, and tetramethyl ammonium hydroxide.

EXAMPLE 1

25 Compounds A and B

Step A: Fermentation of *Trichoderma virens* MF 5783 (ATCC 74180)

Fermentation conditions for the production of Compound A by *Trichoderma virens* MF5783 (ATCC 74180) were as follows. Vegetative mycelia of the culture were prepared by inoculating 54 mL seed medium (Table 1) in a 250 mL unbaffled Erlenmeyer flask with lyophilized mycelia of MF5783. Seed cultures were incubated for 3 days at 25°C and 50% relative humidity on a rotary shaker with a 5-cm throw

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at 220 rpm in a room with constant fluorescent light. Two-mL portions of the 3-day culture were used to inoculate a solid brown rice-based production medium 1 (Table 2) in 250 mL unbaffled Erlenmeyer flasks. The flasks were incubated under static conditions at 25°C with 50% relative humidity in a room with constant fluorescent light: Maximal production of Compounds A and B on the solid based medium occurs by day 21. At harvest, Compounds A and B were extracted from the cultures with methyl ethyl ketone (70 mL per 250 mL flask and shaken at 220 rpm for 1 hour at 25°C).

Step B: Isolation of Compound A and Compound B

The solid fermentation broth (2 L WBE) obtained above (IC₅₀=10 mL WBE per mL in the [¹²⁵I]ChTX binding assay) was extracted with methyl ethyl ketone. The residue was partitioned between hexane (5.6 g) and methanol (95%, 16.4 g). Successive flash chromatography on SiO₂ (methanol-methylene chloride (stepwise elution)) followed by BAKERBOND C₁₈ (40 m; methanol-water) yielded two active fractions, corresponding to Compounds A and B. HPLC of the fraction I on Partisil 10 ODS-3 (22 x 50) using 60% methanol-water (10 ml per min) was carried out to provide Compound B (82 mg; C₁₅H₂₆O₂; M.W. 238.1932 (calcd), 238.1965 (found) IC₅₀ = 200 nM); retention time 90 min). Fraction II was also subjected to HPLC on PARTISIL 10 ODS-3 (22 x 50) using 90% methanol-water to give Compound A (8.5 mg; C₃₃H₅₈O₄; M.W. 518.4334 (calcd), 518.4322 (found); IC₅₀ = 360 nM; retention time 258 min). ¹H NMR (CD₃OD, 300 MHz; only prominent peaks indicated) δ 0.88 (d, J = 7, 3H), 0.90 (t, 3H), 0.95 (d, J = 6.5, 3H), 1.00 (s, 3H), ~1.30 (m), 1.82 (h, J = 7, 2H), 2.32 (t, J = 7, 2H), 4.02 (dd, J = 3, 6.5, 1H), 4.46 (m, 2H), 5.34 (m, 2H), 5.70 (m, 1H). Abbreviations: s = singlet, d= doublet, t = triplet, h = heptet, br = broad, J = ¹H-¹H coupling constant in Hertz (±0.5 Hz). The data were referenced to the solvent peak at δ 3.30 downfield of TMS. ¹³C NMR (CD₃OD, 100 MHz) 14.5, 17.5, 18.2, 21.3, 22.3, 23.8, 26.2, 28.12, 28.14, 30.2 (2 x), 30.3, 30.4, 30.5, 30.6, 30.8, 30.9, 31.7, 33.1, 35.2, 36.0, 43.2, 43.5, 51.2, 59.6, 71.8, 72.1, 85.1, 129.3, 130.8, 130.9,

- 25 -

139.1, 175.4 ppm. The data were referenced to the solvent peak at 49.0 ppm downfield of TMS. The carbon count of 33 is in agreement with the HR-MS derived molecular formula.

5

EXAMPLE 2

Preparation of Compounds D and C

To a solution of Compound B (20 mg, C₁₅H₂₆O₂, 0.08 mM, produced according to the procedures of Example 1) in triethylamine (0.5 mL), were added 4-dimethylaminopyridine (20 mg, 0.16 mM) and oleic anhydride or linoleic anhydride (175 mg, 0.32 mM). The mixture was purged with N₂, sealed, and stirred. The reaction was allowed to proceed at room temperature for 24 h to give Compound D or Compound C, respectively. Ice water (2 mL) was added to the mixture to quench the reaction, and the esters were extracted with ether (2 mL x 3). The organic layer was pooled, washed with saturated NaCl (1 x 2 mL), dried over anhydrous MgSO₄, and filtered through a sintered glass. The esters were purified by HPLC on PARTISIL 10 ODS-3 (22 x 50) using methanol-water (flow rate 10 mL per min; stepwise gradient; 30 min, 80%, 120 min, 90%, and 180 min, 100%). The esters were eluted at 143.6 min (28.0 mg (66.4%)) and 137.5 min (25.9 mg (61.6%)), respectively.

Compound D: C₃₃H₅₈O₃, MS (m/z) 502 (M⁺), 484 (M-18). ¹H NMR (CD₂Cl₂, 300 MHz; only prominent peaks indicated) δ 0.84 (d, *J* = 7, 3H), 0.85 (d, *J* = 7, 3H), 0.99 (s, 3H), ~1.26 (m), 1.81 (h, *J* = 7, 1H), 1.96 (dd, *J* = 8.5, 14.5, 1H), 2.29 (t, *J* = 7.5, 2H), 4.99 (dd, *J* = 2, 8.5, 1H), ~5.32 (m, 2H). Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, qt = quintet, h = heptet, br = broad, *J* = ¹H-¹H coupling constant in Hertz (±0.5 Hz). The data were referenced to the solvent peak at δ 5.32 downfield of TMS. ¹³C NMR (CD₂Cl₂, 100 MHz) 14.3, 17.0, 17.9, 20.9, 21.3, 23.1, 25.4, 27.51 (2 x), 27.55, 29.44, 29.48, 29.52, 29.7 (2 x), 29.9, 30.1, 30.2, 32.3, 34.8, 34.9, 35.5, 42.4, 43.1, 48.0, 58.2, 74.6, 84.6, 122.6, 130.1, 130.3, 139.5, 172.5 ppm. The data were referenced to the solvent peak at 53.8 ppm downfield of TMS.

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The carbon count of 33 is in agreement with the HR-MS derived molecular formula.

Compound C: C₃₃H₅₆O₃, MS (m/z) 500 (M⁺), 482 (M-18). ¹H NMR (CD₂Cl₂, 300 MHz; only prominent peaks indicated) δ 0.85 (d, *J* = 7, 3H), 0.86 (d, *J* = 7, 3H), 0.87 (t, 3H), 1.00 (s, 3H), 1.30 (m), 1.81 (h, *J* = 7, 1H), 1.97 (dd, *J* = 8.5, 14.5, 1H), 2.29 (t, *J* = 7.5, 2H), 2.76 (m, 2H), 5.00 (dd, *J* = 2, 8.5, 1H), ~5.32 (m, 4H). The data were referenced to the solvent peak at δ 5.32 downfield of TMS. ¹³C NMR (CD₂Cl₂, 100 MHz) 14.2, 17.0, 17.9, 20.9, 21.3, 23.0, 25.4, 26.0, 27.51, 27.54 (2 x), 29.44, 29.49, 29.52, 29.7, 30.0, 31.9, 34.8, 34.9, 35.5, 42.4, 43.1, 48.0, 58.2, 74.6, 84.8, 122.6, 128.2, 128.3, 130.3, 130.5, 139.5, 172.5 ppm. The data were referenced to the solvent peak at 53.8 ppm downfield of TMS. The carbon count of 33 is in agreement with the HR-MS derived molecular formula.

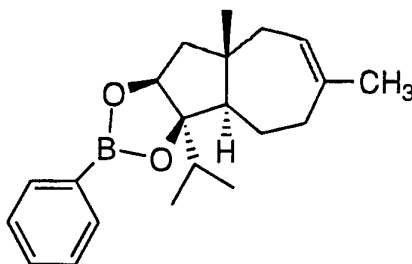
EXAMPLE 3

Preparation of Compound E

Aqueous NaOH (5%, 0.1 mL) was added to an ethanolic solution of Compound A (1.96 mg; 0.0038 mM). The mixture was stirred at room temperature overnight. The solvent was removed, water (1 mL) was added, and the mixture was extracted with CH₂Cl₂ (3 x 1 mL). The pooled solution was washed with saturated NaCl (1 x 1 mL), dried over anhydrous MgSO₄, and the solvent was removed under N₂ to give Compound E. It was purified by HPLC on ULTRACARB 5 ODS (30) (4.6 x 25) using CH₃CN-H₂O (H₂O, 30 min followed by 40% CH₃CN-H₂O; flow rate 1 mL/min; retention time 51.5 min).

Compound E: C₁₅H₂₆O₃ MS (m/z) 236 (M-H₂O, found 236.1777, calculated 236.1776 for C₁₅H₂₄O₂) ¹H NMR (CD₂Cl₂, 300 MHz; only prominent peaks indicated) δ 0.87 (d, *J* = 7, 3H), 0.95 (d, *J* = 7, 3H), 1.00 (s, 3H), 1.60 (d, *J* = 4.5, 1H), 1.81 (h, *J* = 7, 1H), 2.11 (dd, *J* = 9, 14.5, 1H), 2.24 (m, 1H), 2.38 (d, OH, *J* = 5.5, 1H), 3.96 (d, *J* = 6, 2H), 4.03 (q, *J* = ~5, 1H), 5.61 (m, 1H). The data were referenced to the solvent peak at δ 5.32 downfield of TMS.

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EXAMPLE 4Preparation of

To an ethereal solution of Compound B was added an excess of phenylboric acid (phenylboronic acid) in ether at room temperature. The mixture was stirred for 30 minutes. The reaction was monitored by TLC (SiO₂; 5% methanol in methylene chloride; R_f: 0.56 for Compound B and 0.88 for the title compound, Compound B phenylborate).

15

EXAMPLE 5Potassium Channel Agonist Activity: Electrophysiological Experiments

20 Patch clamp recordings of currents flowing through large-conductance calcium-activated potassium (maxi-K) channels were made from membrane patches excised from cultured bovine aortic smooth muscle cells using conventional techniques (Hamill *et al.*, 1981, Pflügers Archiv 391, 85-100) at room temperature. Glass capillary tubing

25 (GARNER #7052) was pulled in two stages to yield micropipettes with tip diameters of approximately 1-2 microns. Pipettes were typically filled with solutions containing (mM): 150 KCl, 10 Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 Mg, 0.01 Ca, and adjusted to pH 7.20 with 3.7 mM KOH. After forming a high resistance (>10⁹ ohms) seal

30 between the sarcolemmal membrane and the pipette, the pipette was withdrawn from the cell forming an excised inside-out membrane patch. The patch was excised into a bath solution containing (mM): 150 KCl, 10 Hepes, 5 EGTA (ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), sufficient Ca to yield a free Ca concentration of 1-5 μM,

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and the pH was adjusted to 7.2 with 10.5 KOH. For example, 4.568 mM Ca was added to give a free concentration of 2 μ M at 22°C. An Axopatch 1C amplifier (Axon Instruments, Foster City, CA.) with a CV-4 headstage was used to control the voltage and to measure the currents
5 flowing across the membrane patch. The input to the headstage was connected to the pipette solution with a Ag/AgCl wire, and the amplifier ground was connected to the bath solution with a Ag/AgCl wire covered with a tube filled with agar dissolved in 0.2 M KCl. Maxi-K channels were identified by their large single channel conductance (~250 pS) and
10 sensitivity of channel open probability to membrane potential and intracellular calcium concentration.

Planar lipid bilayers were formed from a solution of 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-oleoylphosphatidylcholine (POPC) in a 7/3 molar ratio dissolved in
15 decane (50 mg/mL). This lipid solution was painted across a small hole (250 micron) separating two aqueous compartments and readily formed bilayers with capacitances of 200-250 pF. The solution on the side that the membranes were added (cis) contained (mM): 150 KCl, 10 HEPES, 0.01 Ca, 3.7 KOH, pH 7.20. The solution on the other side (trans)
20 contained (mM): 25 KCl, 10 HEPES, 0.01 Ca, 3.7 KOH, pH 7.20. Plasma membrane vesicles purified from bovine aortic smooth muscle (Slaughter *et al.* 1989, *Biochemistry* 28:3995-4002) were added to the cis side until channel incorporation occurred. After channel incorporation, the concentration of KCl on the trans side was increased to 150 mM to
25 prevent further channel incorporation. The orientation of maxi-K channels after insertion into the bilayer was determined from the calcium and voltage sensitivity of the channel. Increases in calcium or voltage on the intracellular side lead to increases in channel open probability. An Axopatch 1C with a CV-4B headstage was used to control the membrane
30 potential and record currents flowing across the bilayer. The inputs to the amplifier were connected to Ag/AgCl wires which connected to the two sides of the bilayer chamber through small tubes filled with agar dissolved in 0.2 M KCl. Experiments were done at room temperature.

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Data was stored on a RACAL STORE 4DS FM tape recorder (Racal Recorders, Vienna, Va.) or on digital video tape using a video cassette recorder after digitizing the signal with VR-10 (Instrutech Corp., Belmont N.Y.) PCM video encoder. The signal was also recorded
5 on chart paper with a GOULD 2400S chart recorder (Gould Inc., Cleveland OH.). For quantitative analysis, the data was played into a DEC 11-73 (Digital Equipment Corp., Maynard, Ma.) after digitization with a DT2782-8D1A analogue to digital converter (Data Translation Inc., Marlboro, Ma.), or played into a MAC IIx or QUADRA 700
10 computer (Apple Computers) after digitization with an ITC-16 interface (Instrutech Corp., Belmont, N.Y.).

The effects of Compound A on maxi-K channels were examined in excised inside-out membrane patches and in lipid bilayers. In patch clamp experiments, 10 μ M Compound A caused clear increases
15 in channel open probability that developed slowly over 5-10 minutes. Specifically, 10 μ M Compound A caused a 2.1 fold increase in channel open probability. These increases in channel open probability were slowly reversed after long (20-60 minutes) washout. In lipid bilayer experiments, addition of 10 μ M Compound A to the outside face of the
20 channel did not increase channel open probability. In contrast, 10 μ M Compound A to the inside face of the channel caused a 1.4 fold increase in channel open probability, suggesting that this compound is more effective when added to the inside than the outside.

The effects of four related compounds, Compound D,
25 Compound C, Compound E and Compound B on Maxi-K channels were investigated in excised, inside-out patch clamp recordings. Application of 10 μ M Compound C or Compound D to the intracellular side of the channel caused small decreases in channel open probability. Application of 10 μ M Compound E to the intracellular side had no observable effect
30 on channel open probability. Compound B had no clear effect on channel open probability at 10 μ M.

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EXAMPLE 6Biochemical Experiments

The interaction of [¹²⁵I]ChTX (Charybdotoxin) with bovine
5 aortic sarcolemma membrane vesicles was determined under conditions
as described in Vazquez *et al.*, 1989, *J. Biol. Chem.* 264:20902-20909.
Briefly, sarcolemma membrane vesicles were incubated in 12x75
polystyrene tubes with ca. 25 pM [¹²⁵I]ChTX (2200 Ci/mmol), in the
absence or presence of test compound, in a media consisting of 20 mM
10 NaCl, 20 mM Tris-HCl pH 7.4, 0.1% bovine serum albumin, 0.1%
digitonin. Nonspecific binding was determined in the presence of 10 nM
ChTX. Incubations were carried out at room temperature until ligand
binding equilibrium is achieved at ca. 90 min. At the end of the
incubation period, samples were diluted with 4 mL ice-cold 100 mM
15 NaCl, 20 mM HEPES-Tris pH 7.4 and filtered through GF/C glass fiber
filters that have been presoaked in 0.5% polyethylenimine. Filters were
rinsed twice with 4 mL ice-cold quench solution. Radioactivity
associated with filters was determined in a gamma counter. Specific
binding data in the presence of each compound (difference between total
20 binding and nonspecific binding) was assessed relative to an untreated
control.

Compound A caused a concentration-dependent inhibition of
[¹²⁵I]ChTX binding. The concentration of compound needed to cause
50% inhibition of binding is ca. 360 nM. Compound D and Compound C
25 were tested at three different concentrations: 1, 10 and 100 μM.
Compound D caused a significant inhibition of binding when tested at
100 μM. At this concentration, binding was 37% of that under control
conditions. Compound C had no effect on binding at concentrations up
to 100 μM. Compound E did not produce any significant effect on toxin
30 binding in the range of concentrations from 1 nM to 100 μM. Compound
B caused a concentration-dependent inhibition of [¹²⁵I]ChTX binding
with half-maximal inhibition taking place at a concentration of 200 nM.

EXAMPLE 7Oral Formulation

5 As a specific embodiment of an oral composition of a potassium channel agonist of the present invention, 100 mg of Compound A is formulated with a sufficient amount of finely divided lactose to provide a total amount of 580 to 590 mg to fill a size 0 hard gelatin capsule.

10 The previous examples are non-limiting and clearly demonstrate that the compounds as claimed in the present application are useful as either Maxi-K potassium channel agonists or as synthetic intermediates in the production of said agonists. In addition, the claimed invention relates to novel microbiological processes for producing the claimed compounds.

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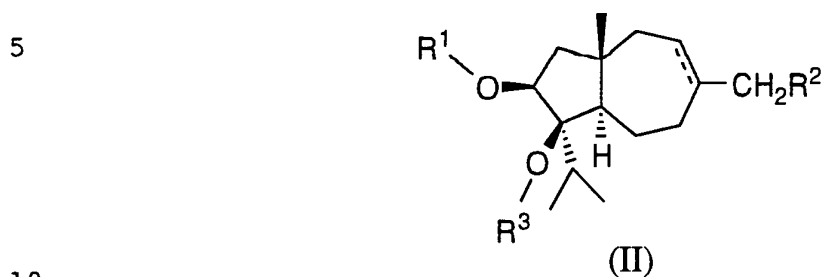
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WHAT IS CLAIMED IS:

1. A compound of the formula:



and its pharmaceutically acceptable salts, wherein:

R¹ is:

- 15
- (a) H,
 - (b) C₁-5alkyl,
 - (c) C₂-5 alkenyl,
 - (d) C₁-30alkylcarbonyl,
 - (e) C₂-30 alkenylcarbonyl,
 - (f) R⁴-O-R⁴-,
 - 20 (e) substituted R⁴-O-R⁴- wherein the substituent is phenyl or methoxy,
 - (f) tetrahydropyran, or
 - (g) (R⁴)₃ Si-R⁴- wherein R⁴ is independently C₁-5alkyl or C₂-5 alkenyl at each occurrence,

25 R³ is:

- (a) H,
- (b) C₁-5alkyl,
- (c) C₂-5 alkenyl,
- (d) C₁-30alkylcarbonyl,
- 30 (e) C₂-30alkenylcarbonyl,
- (e) R⁴-O-R⁴-,
- (f) substituted R⁴-O-R⁴- wherein the substituent is phenyl or methoxy,
- (g) tetrahydropyran, or

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(h) $(R^4)_3 SiR^4$,
 or R^1 and R^3 together with the adjacent oxygen and ring carbon atoms,
 form a five-membered ring wherein R^1 and R^3 together are a single
 substituted or unsubstituted carbon or boron atom, wherein the carbon
 atom is substituted with two substituents independently selected from:

- 5
 (a) H,
 (b) C₁₋₄ alkyl,
 (c) C₂₋₅ alkenyl,
 (d) C₁₋₆ alkoxy,
 10 (e) C₂₋₆ alkenyloxy, and
 (f) phenyl, and

wherein the boron atom is substituted with one substituent selected from:

- (a) H,
 (b) C₁₋₄ alkyl,
 15 (c) C₂₋₅ alkenyl,
 (d) C₁₋₆ alkoxy,
 (e) C₂₋₆ alkenyloxy, and
 (f) phenyl;

R^2 is:

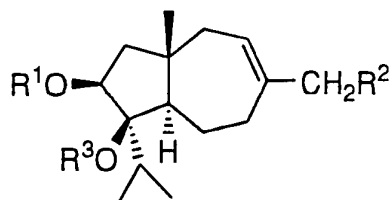
- 20 (a) H,
 (b) OH,
 (c) C₁₋₅alkyloxy,
 (d) C₂₋₅ alkenyloxy,
 (e) C₁₋₃₀alkylcarbonyloxy,
 25 (f) C₂₋₃₀alkenylcarbonyloxy,
 (g) R^4-O-R^4-O- ,
 (h) substituted R^4-O-R^4-O- wherein the substituent is phenyl or
 methoxy,
 (i) tetrahydropyran-oxy, or
 30 (j) $(R^4)_3 Si-R^4-$,

provided that when R^2 is H and R^3 is H, R^1 is not H or CH_3CO ;
 R^4 is independently C₁₋₅alkyl or C₂₋₅ alkenyl at each occurrence, and
 the dashed line indicates the optional presence of a double bond.

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2. The compound according to Claim 1 of the formula:

5



wherein:

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R¹ is:

- (a) H,
- (b) CH₃,
- (c) C₁-30alkylcarbonyl,
- (d) C₂-30alkenylcarbonyl,
- 15 (e) CH₃OCH₂,
- (f) PhCH₂OCH₂,
- (g) t-BuOCH₂,
- (h) CH₃OCH₂CH₂OCH₂,
- (i) tetrahydropyran, or
- 20 (j) Me₃SiCH₂CH₂;

R² is:

- (a) H,
- (b) OH,
- (c) methoxy,
- 25 (d) C₁-30alkylcarbonyloxy,
- (e) C₂-30alkenylcarbonyloxy,
- (f) CH₃OCH₂O,
- (g) PhCH₂OCH₂O,
- (h) t-BuOCH₂O,
- 30 (i) CH₃OCH₂CH₂OCH₂O,
- (j) tetrahydropyranoxy, or
- (k) Me₃SiCH₂CH₂O,

provided that when R² is H and R³ is H, R¹ is not H or CH₃CO;
and

- 35 -

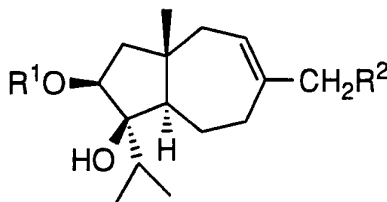
R³ is:

- 5 (a) H,
 (b) C₁₋₅alkyl,
 (c) C₂₋₅ alkenyl,
 (d) C₁₋₃₀alkylcarbonyl,
 (e) C₂₋₃₀alkylcarbonyl,
 (f) R⁴-O-R⁴-,
 (e) substituted R⁴-O-R⁴- wherein the substituent is phenyl or
 methoxy,
 10 (f) tetrahydropyran, or
 (g) (R⁴)₃ SiR⁴, and

R⁴ is independently C₁₋₅alkyl at each occurrence.

3. The compound according to Claim 1 of the formula:

15



20

wherein:

R¹ is:

- 25 (a) H,
 (b) CH₃,
 (c) C₁₋₃₀alkylcarbonyl,
 (d) C₂₋₃₀alkenylcarbonyl,
 (e) CH₃OCH₂,
 30 (f) PhCH₂OCH₂,
 (g) t-BuOCH₂,
 (h) CH₃OCH₂CH₂OCH₂,

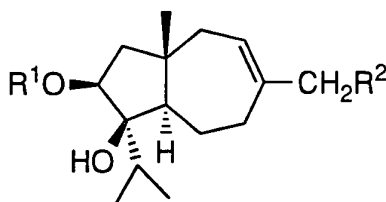
R² is:

- (a) H,

- 36 -

- (b) OH,
 (c) methoxy,
 (d) C₁-30alkylcarbonyloxy,
 (e) C₂-30alkenylcarbonyloxy,
 (f) CH₃OCH₂O,
 (g) PhCH₂OCH₂O,
 (h) t-BuOCH₂O,
 (i) CH₃OCH₂CH₂OCH₂O,
 provided that when R² is H, R¹ is not H or CH₃CO.

4. The compound according to Claim 1 of the formula:



wherein:

R¹ is:

- (a) H,
 (b) CH₃,
 (c) C₁-30alkylcarbonyl,
 (d) C₂-30alkenylcarbonyl,
 (e) CH₃OCH₂,
 (f) PhCH₂OCH₂,
 (g) t-BuOCH₂,
 (h) CH₃OCH₂CH₂OCH₂,

R² is:

- (a) H,
 (b) methoxy,
 (c) C₁-30alkylcarbonyloxy,
 (d) C₂-30alkenylcarbonyloxy,

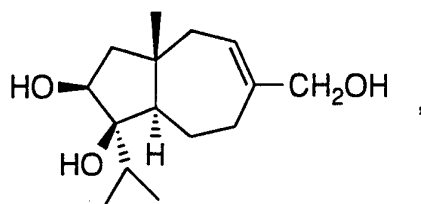
- 37 -

- (e) $\text{CH}_3\text{OCH}_2\text{O}$,
 (f) $\text{PhCH}_2\text{OCH}_2\text{O}$,
 (g) $t\text{-BuOCH}_2\text{O}$,
 (h) $\text{CH}_3\text{OCH}_2\text{CH}_2\text{OCH}_2\text{O}$,
 5 provided that when R^2 is H, R^1 is not H or CH_3CO .

5. The compound according to Claim 3 of the formula:

(a)

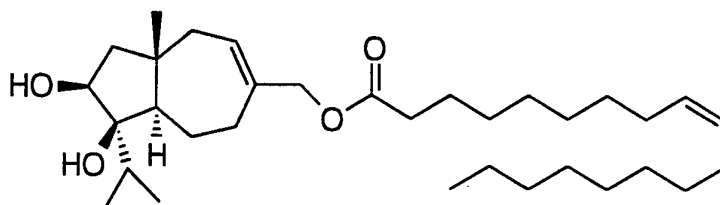
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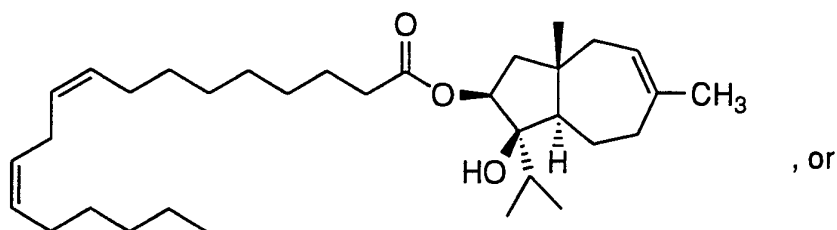
(b)

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(c)

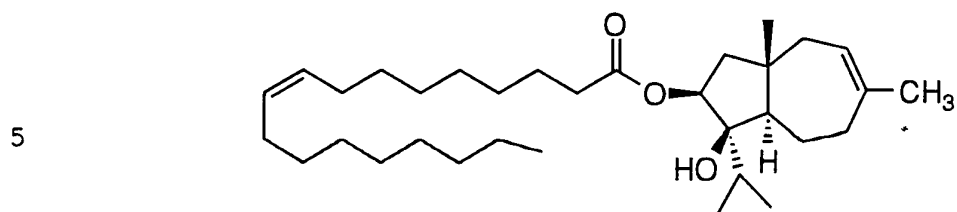
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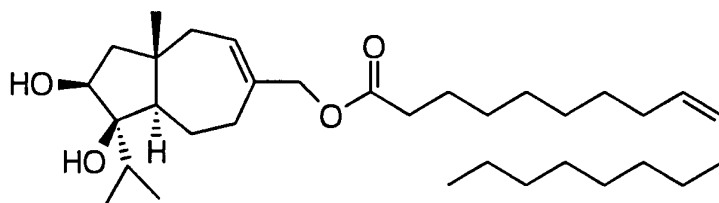
- 38 -

(d)



6. The compound according to Claim 3 of the formula:

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7. A method of activating a potassium channel in a mammal, including a human, comprising administering a pharmacologically effective amount of a compound as claimed in Claim 4.

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8. A method of eliciting hyperpolarization of a mammalian cell wherein the cell contains a potassium channel comprising administration of a pharmacologically effective amount of a potassium channel agonist as claimed in Claim 4.

25

9. A method of treating neuronal diseases such as ischemia and convulsions comprising the administration to a subject in need of such treatment of a pharmacologically effective amount of a potassium channel agonist as claimed in Claim 4.

30

10. A method of treating hypertension comprising the administration to a subject in need of such treatment of a

- 39 -

pharmacologically effective amount of a potassium channel agonist as claimed in Claim 4.

5 11. A method of treating asthma comprising the administration to a subject in need of such treatment of a pharmacologically effective amount of a potassium channel agonist as claimed in Claim 4.

10 12. A method of treating urinary incontinence comprising the administration to a subject in need of such treatment of a pharmacologically effective amount of a potassium channel agonist as claimed in Claim 4.

15 13. A method of treating dysmenorhea comprising the administration to a subject in need of such treatment of a pharmacologically effective amount of a potassium channel agonist as claimed in Claim 4.

20 14. A method of treating urinary incontinence comprising the administration to a subject in need of such treatment of a pharmacologically effective amount of a potassium channel agonist as claimed in Claim 4.

25 15. A method of treating irritable bowel syndrome comprising the administration to a subject in need of such treatment of a pharmacologically effective amount of a potassium channel agonist as claimed in Claim 4.

30 16. A pharmaceutical composition comprising a compound according to Claim 4 and a pharmaceutically acceptable carrier.

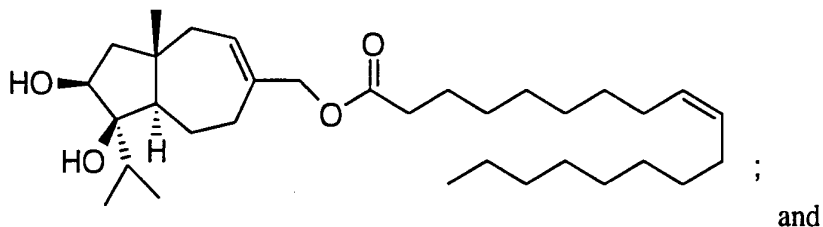
17. A pharmaceutical composition for activating a calcium-activated potassium channel in a mammal, including a human,

- 40 -

comprising a compound as claimed in Claim 4 and a pharmaceutically acceptable carrier.

18. A method of producing a compound according to Claim 1, comprising:

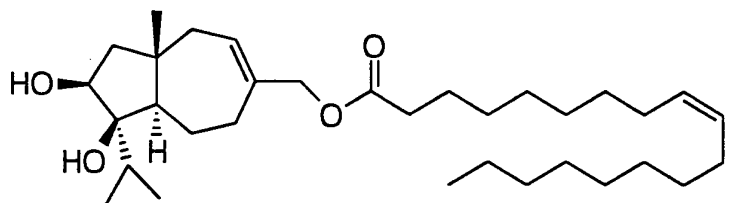
(a) culturing the fungal organism *Trichoderma virens* (ATCC 74180) so that the fungal organism produces the compound of the formula:



(b) purifying the compound produced in step (a) to a substantially purified form.

19. A method of producing a compound according to Claim 1, comprising:

(a) culturing the fungal organism *Trichoderma virens* (ATCC 74180) so that the fungal organism produces the compound of the formula:

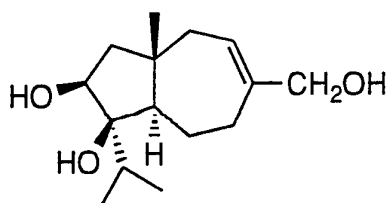


(b) purifying the compound produced in step (a) to a substantially purified form; and

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(c) optionally reacting the purified product of step (b) with a base to produce a compound of the formula:

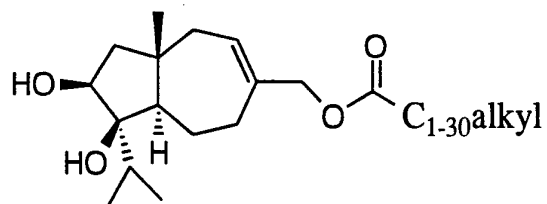
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which is further reacted after suitable protection of the vicinal hydroxyl groups with a compound selected from a C₁-30 alkyl anhydride or a C₁-30 alkyl halide and then deprotected to produce a compound with potassium channel agonist activity of the formula:

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20. A biologically pure culture of *Trichoderma virens*, ATCC 74180.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14491

<p>A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : A61K 31/015, 31/12, 31/13; C07C 35/20, 35/21, 53/132; C12N 1/14 US CL : Please See Extra Sheet. According to International Patent Classification (IPC) or to both national classification and IPC</p>												
<p>B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/945; 514/660, 691, 763, 826, 899, 906, 929; 554/229; 568/816, 817, 819, 821</p>												
<p>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched</p>												
<p>Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, CAS ONLINE</p>												
<p>C. DOCUMENTS CONSIDERED TO BE RELEVANT</p>												
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.										
A	Journal of Natural Products, volume 53, no. 5, issued Sept-Oct 1990, N. Watanabe et al., "CAF-603: A New Antifungal Carotane Sesquiterpene. Isolation and Structure Elucidation", pages 176-1181.	1-20										
A	JP, A, 61-130250 (Watanabe et al.) 18 June 1984, entire document.	1-20										
<p><input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.</p>												
<p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>*A* document defining the general state of the art which is not considered to be of particular relevance</td> <td>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>*E* earlier document published on or after the international filing date</td> <td>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>*O* document referring to an oral disclosure, use, exhibition or other means</td> <td>*Z* document member of the same patent family</td> </tr> <tr> <td>*P* document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			*A* document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	*E* earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	*O* document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family	*P* document published prior to the international filing date but later than the priority date claimed	
A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention											
E earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone											
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art											
O document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family											
P document published prior to the international filing date but later than the priority date claimed												
<p>Date of the actual completion of the international search 23 MARCH 1995</p>		<p>Date of mailing of the international search report 06 APR 1995</p>										
<p>Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230</p>		<p>Authorized officer DEBORAH D. CARR Telephone No. (703) 308-1235</p>										

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/14491

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14491

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/945; 514/660, 691, 763, 826, 899, 906, 929; 554/229; 568/816, 817, 819, 821

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-8 and 16-20, drawn to compound, composition, method of making, method of using.

Group II, claim 9, drawn to treating neuronal diseases.

Group III, claim 10, drawn to treating hypertension.

Group IV, claim 11, drawn to treating asthma.

Group V, claims 12 and 14, drawn to treating urinary incontinence.

Group VI, claim 13, drawn to treating dysmenorrhea.

Group VII, claim 15, drawn to treating irritable bowel.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Applicant is only allowed the first method of use/making if multiple inventions are disclosed. 37 CFR 1.475(d)