A NOVEL INOSITOL MONO-PHOSPHATASE INHIBITOR FROM *Memnoniella echinata*

PRODUCING ORGANISM, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND *IN VITRO* BIOLOGICAL PROPERTIES[†]

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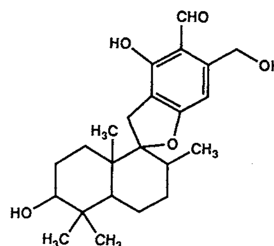
A novel inositol mono-phosphatase inhibitor, L-671,776 (**1**), was discovered from a culture of the hyphomycete, *Memnoniella echinata* (ATCC 20928). **1** has a molecular weight of 388 and a molecular formula of $C_{23}H_{32}O_5$. The mode of inhibition is non-competitive, with a *Ki* of 450 μM. It shows no inhibition of *myo*-inositol 1,4-bisphosphate 1-phosphatase or *myo*-inositol 1,4,5-triphosphate 5-phosphatase, although it weakly inhibits *myo*-inositol 1,4,5-triphosphate 3-kinase (IC_{50} = 3 mM). It elevates inositol monophosphates in rat parotid slices (EC_{50} approximately 3 mM), but abolishes agonist effects. It also produces short-lived contraction of guinea pig trachea at 300 μM.

In the phosphoinositide cycle, *myo*-inositol mono-phosphatase (EC 3.1.3.25) (IPlase) is a key enzyme responsible for the provision of inositol for phosphoinositide synthesis either from glucose *via* inositol-3-phosphate or from hydrolysis of inositol polyphosphates *via* inositol-1-phosphate, inositol-3-phosphate and inositol-4-phosphate, all of which are substrates. Thus, inhibitors of this enzyme may be useful tools for modulating cell signalling through phosphoinositide turnover. In our search for inositol mono-phosphatase inhibitors from microbes, we encountered a novel sesquiterpene, L-671,776 (**1**) (Fig. 1), which may be classified as a triprenyl phenol of K-76 type[1]. In this report we will present the identification of the producer, fermentation, isolation, physico-chemical and biological properties of this compound.

Fig. 1. Structure of L-671,776 (**1**).



Description of Producing Organism

The producing organism (MF5195, ATCC 20928) was recovered from a soil sample collected from Bosna's Pass, Namibia. It exhibited all the essential and diagnostic features of the species,

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Memnoniella echinata (Rivolta) Galloway, as described by JONG and DAVIS[2] and DOMSCH *et al.*[3]. A summary of these significant morphological features of our strain is included in the following description.

Colonies on corn meal agar effuse, hyaline at edges, soon darkening to dark grayish olive, Dark Olive, Dark Olive-Gray, Olive Gray, Iron Gray, Olivaceous Black, to Blackish Mouse Gray (capitalized color names from RIDGWAY[4]), sparse to velvety, with stroma and setae absent, composed of subaerial, appressed to slightly immersed mycelium. Mycelium composed of septate, branched, straight, flexuous, to contorted hyphae; hyphae hyaline to olive-gray in water, $1 \sim 4\,\mu m$ in diameter.

Conidiophores macronematous, erect, determinate, unbranched straight to slightly curved, arising at right angles from a foot cell, 0- to 3-septate, $22 \sim 30\,\mu m \times 3.5 \sim 5\,\mu m$, attenuated toward the apex, with a slight apical swelling; walls roughened due to granular pigmented surface, pale olive-gray to olive brown in water.

Conidiogenous cells phialidic, enteroblastic, discrete, reniform, in terminal whorls of $4 \sim 7$, $8.5 \sim 11.5 \times 3.5 \sim 6\,\mu m$, without conspicuous collarettes.

Conidia globose to subglobose, rarely broadly elliptical in side view, $5.5 \sim 7 \times 4.5 \sim 6.5\,\mu m$, with punctate-verrucose ornamentation, usually biguttulate, olive-gray to grayish brown in water when mature, adhering in slimy masses at apices of young conidiogenous cells, soon drying and adhering together in chains.

Memnoniella echinata is a common and widely distributed saprobic hyphomycete that occurs in soils and on a variety of organic substrata. Based on results from our screening program, inositol mono-phosphatase inhibition by **1** or **1**-like compounds appears to be wide spread among strains of *Memnoniella* and the very similar genus, *Stachybotrys*. During the course of screening soil fungi for this activity, members of these genera repeatedly produced activity in our assay. In addition to the strain of *M. echinata* mentioned above, we identified another strain of *M. echinata* (MF3686, from cordage, India) a strain of *Stachybotrys cylindrospora* Jensen (MF5277, from soil, Aurangabad, India), and a strain of *Stachybotrys chartarum* (Ehrenberg) S. J. hughes (MF5217, from soil, Lamphun, Thailand) that produced **1**.

Fermentation

A 2 ml portion of a frozen culture ATCC 20928 in glycerol was defrosted and aseptically transferred to a 250-ml unbaffled Erlenmeyer flask containing 50 ml of sterile seed medium containing (per liter, pH 6.8) corn steep liquor (5 g), tomato paste (40 g), oat flour (10 g), glucose (10 g) and trace element mix (10 ml). The trace element mix contains (per liter) $FeSO_4 \cdot 7H_2O$ (1 g), $MnSO_4 \cdot 4H_2O$ (1 g), $CuCl_2 \cdot 2H_2O$ (25 mg), $CaCl_2$ (100 mg), H_3BO_3 (56 mg), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (19 mg) and $ZnSO_4 \cdot 7H_2O$ (200 mg). The mixture was incubated at 28°C on a 220 rpm, 5 cm throw shaker for 3 days.

Approximately two percent inoculum was used to inoculate 2-liter Erlenmeyer flasks each containing 500 ml of the seed medium. The second stage seed flasks were incubated at 28°C for 24 hours on a rotary shaker at 220 rpm.

The production medium was adjusted to pH 7.0 with NaOH prior to sterilization. Four 14-liter and two 22-liter scale vessels containing sterile medium (10 and 15 liters working volume, respectively) consisting of (per liter) glycerol (85 g), corn steep liquor (5 ml), corn meal (10 g), lard water (5 g), soybean meal (5 g), cod liver oil (2 ml), tomato paste (5 g), glycine (2 g) and P-2000 antifoam (Dow, 2 ml). Five percent inoculum was then inoculated into each fermenter. The fermentations were carried out at 25°C under a range of conditions for airflow ($2 \sim 5$ liters/minute) and agitation rate ($200 \sim 500$ rpm) for $96 \sim 118$ hours.

Isolation and Purification

Eighty liters of fermentation were premixed with Celite (4 kg), extracted with methylethylketone (96 liters), and filtered over a layer of Celite and Whatman No. 3 filter paper. The upper organic layer of the filtrate was flash evaporated (under reduced pressure and $<35°C$) to give 183 g of crude extract. Differential pH partition was then performed. The crude extract was redissolved in methylethylketone (2.4 liters) and washed with saturated $NaHCO_3$ (aq) (2 liters). The organic layer was then extracted with 1 M NaOH (aq) (2×1.5 liters). The organic layer containing neutrals and bases was flash evaporated to a dry weight of 133.13 g. The aqueous layer was immediately neutralized with conc HCl and extracted with methylethylketone (3.6 liters). The phenolic fraction was then flash evaporated to a dry weight of 34.35 g. Silica gel column chromatography of this phenolic extract (E. Merck, Silica gel 60, $40 \sim 63$ µm, $V_b = 2,450$ ml) using 40% Me_2CO - hexanes as the mobile phase at a flow rate of 50 ml/minute was performed. Fractions of 200 ml each were collected. Analytical TLC monitoring of effluent using the same mobile phase suggested the pooling of fractions 25 to 37 and 9 g of **1** was recovered (Rf = 0.35). Analytical HPLC (column: Whatman Partisil 5 ODS-3 0.46×10 cm; mobile phase: acetonitrile - H_2O, 40 : 60 (v/v) at 1 ml/minute; detection: UV at 215 nm) also revealed homogeniety of this material with $k' = 6.9$.

Physico-chemical Properties

Mass spectral data were recorded on a Finnigan-MAT 212 mass spectrometer in the electron impact mode (90 eV). High resolution exact mass measurements were performed on the same instrument using the peak matching method with perfluorokerosene as the internal standard. Trimethylsilyl (TMS) derivatives were formed using a 1 : 1 mixture of bistrimethylsilyltrifluoroacetamide (BSTFA) - pyridine at 50°C for 10 minutes.

The critical ions observed in the EI mass spectrum of the underivatized compound are listed in Table 1. **1** has the molecular weight 388, with the molecular formula $C_{23}H_{32}O_5$, and forms a tri-TMS derivative. The major fragment ions are *m/z* 180 (forms a di-TMS derivative) corresponding to an empirical formula of $C_9H_8O_4$ and 207 (forms a mono-TMS derivative) corresponding to an empirical formula of $C_{14}H_{23}O$. These two fragment ions describe the main parts of the compound, the 180 fragment corresponds to a substituted aromatic ring and the 207 portion can be described as an aliphatic chain.

NMR spectra were recorded at 21°C on a Varian XL-400 NMR spectrometer in CD_3CN. Chemical shifts are reported in ppm relative to TMS. The ^1H NMR spectrum of **1** is shown in Fig. 2. Its ^{13}C NMR spectrum displayed resonances at δ 15.9, 16.4, 21.7, 22.7, 24.8, 25.9, 28.8, 31.3, 32.0, 37.7, 38.3, 41.0, 43.2, 64.2, 75.6, 100.9, 109.2, 110.6, 113.3, 146.2, 159.9, 169.9, and 189.5 ppm. This carbon data, including a "gated" decoupled spectrum (not shown) indicated the presence of 23 carbons and 29 carbon bound protons, corroborating with the empirical formula, $C_{23}H_{32}O_5$, obtained by MS. The assigned carbon types included $4 \times CH_3$, $5 \times CH_2$, $1 \times CH_2O$, $2 \times CH$, $1 \times CHO$, $2 \times$ -C-, $1 \times$ -C-O or -C-, $5 \times$ -C=, $1 \times$ HC= and $1 \times$ HC=O.

The observed UV maxima for this compound (230, 286 and 330 nm with E % 230, 187 and 118, respectively) are consistent with the triple maxima generally observed in *o*-hydroxybenzaldehydes and

Table 1. Critical ions observed in the EI-MS of L-671,776.

Found mass (*m/z*)	Calculated	Formula
388.2268	388.2250	M^+, $C_{23}H_{32}O_5$
370	—	$M-H_2O$
207.1754	207.1749	$C_{14}H_{23}O$
189.1661	189.1643	$C_{14}H_{21}$
180.0458	180.0423	$C_9H_8O_4$
175.1503	175.1487	$C_{13}H_{19}$
135.1180	135.1174	$C_{10}H_{15}$

Fig. 2. ^1H NMR spectrum of L-671,776 in CD_3CN at 400 MHz.

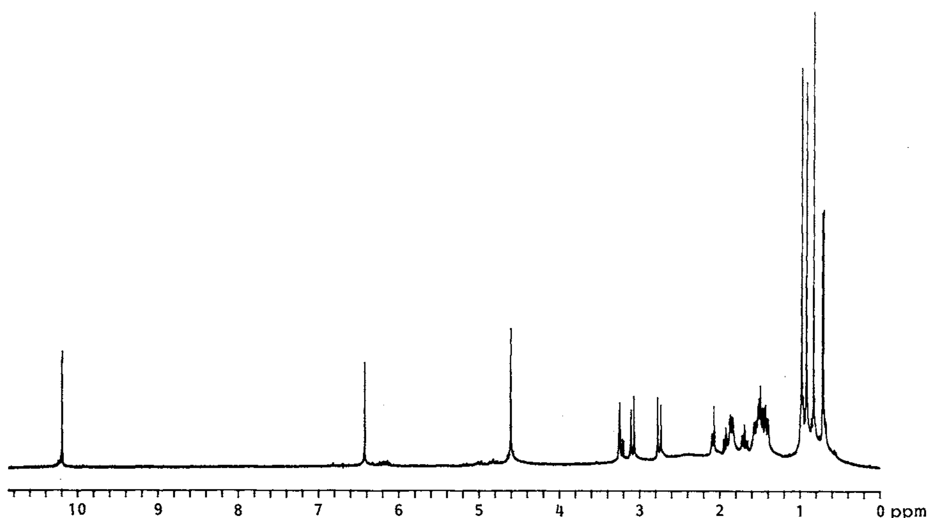


Fig. 3. Dose-response curve of L-671,776 in inhibition of bovine IPlase in the presence of 0.1 mM (●) and 1 mM (○) L-*myo*-inositol-1-phosphate.

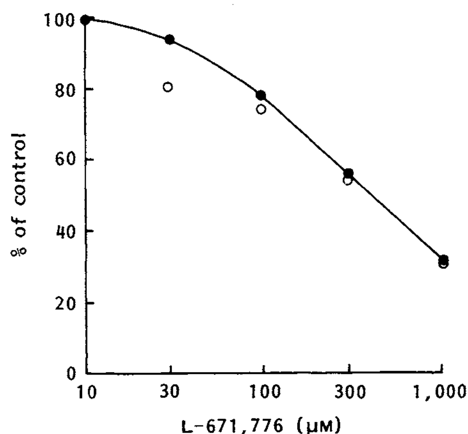


Table 2. Specificity of L-671,776 (**1**) as a phosphatase inhibitor.

Enzyme	IC_{50} of **1** (mM)
Myo-inositol mono-phosphatase[a]	0.40
Myo-inositol 1,4-bisphosphate 1-phosphatase[a]	>10
Myo-inositol 1,4,5-triphosphate 5-phosphatase[b]	>10
Myo-inositol 1,4,5-triphosphate 3-kinase[c]	3

[a] Partially purified bovine cerebral cortex preparation.
[b] Washed rat cerebral cortical membrane preparation.
[c] Rat brain supernatant fraction.

o-hydroxyacetophenones. Its IR (KBr) spectrum displayed absorptions at 3440, 2870, 2930, 1650, 1610 cm^{-1}.

Based on these observations, **1** was classified as a triprenyl phenol of K-76 type with the structure shown. Details of structure determination will be described elsewhere.

In Vitro Biological Properties

Myo-inositol mono-phosphatase (EC 3.1.3.25) (IP1ase) and *myo*-inositol 1,4-bisphosphate (Ins(1,4)P_2) 1-phosphatase activities were performed as described by GEE *et al.*[5] and RAGAN *et al.*[6] using a partially purified bovine cerebral cortex preparation[7] as an enzyme source. For IP1ase activity measurement, ^{14}C-labeled L-*myo*-inositol-1-phosphate was used as a substrate. Ins(1,4)P_2 1-phosphatase was monitored using D-[^3H]Ins(1,4)P_2 as substrate. *Myo*-inositol 1,4,5-triphosphate (Ins(1,4,5)P_3) 5-phosphatase was assayed using a washed rat cerebral cortical membrane preparation as described by ERNEUX *et al.*[8]. Ins(1,4,5)P_3 3-kinase was assayed using a rat brain supernatant fraction as described by JOHANSON *et al.*[9]. Activities were measured using [^3H]substrates. Substrate and product were separated on Dowex-formate columns.

A dose-response curve of **1** in the inhibition of bovine IP1ase in the presence of 0.1 and 1 mM L-*myo*-inositol-1-phosphate is depicted in Fig. 3. The mode of inhibition is non-competitive (Ki=450 μM), since there is no effect on IC_{50} of an increase in substrate concentration. The mode of inhibition was also

Fig. 4. Elevation of rat parotid inositol monophosphates by L-671,776.

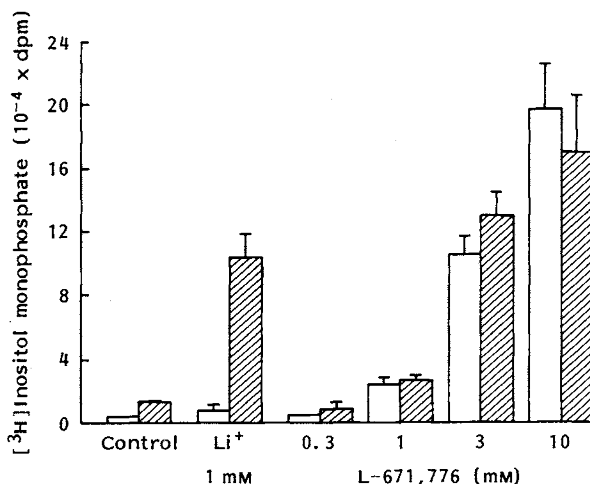
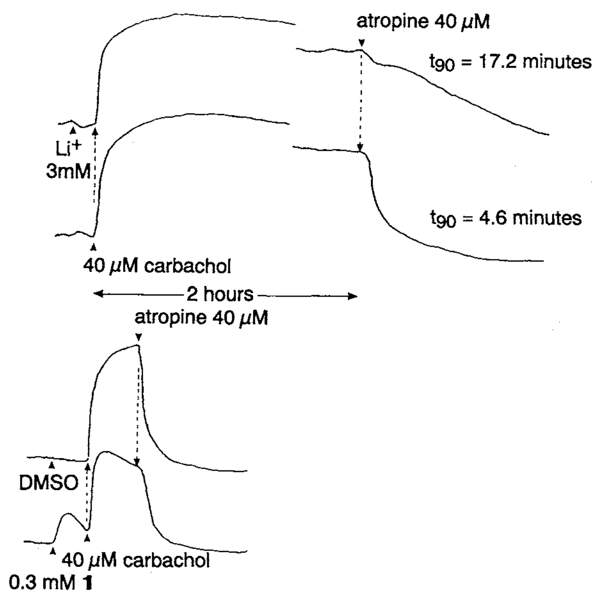


Fig. 5. Effect of L-671,776 (**1**) on guinea pig trachea.



a) The addition of carbachol (40 μM) produces a powerful contraction of the trachea (2-3 gf) that is well maintained in the presence of 5 μM indomethacin. When 3 mM LiCl is present, the rate of offset (t_{90}) upon addition of atropine (40 μM) is much prolonged (top) compared to a control tissue.

b) The addition of 0.3 mM **1** (bottom) produced a small contraction of the trachea and reduced the amplitude and maintenance of the response to a subsequent dose of 40 μM carbachol. Addition of 40 μM atropine produced a rapid relaxation of the control preparation to baseline level (top). This rate was not prolonged in the presence of **1**.

non-competitive with respect to Mg^{2+} concentration (data not shown). As a comparison, Li^+ is an uncompetitive inhibitor of bovine IP1ase[5]. **1** is therefore neither a Li^+ mimic, nor substrate analog nor interferes with Mg^{2+} binding. In spite of this, **1** is quite specific in its inhibition of phosphatases. At 3 mM, **1** did not inhibit either Ins(1,4)P_2 1-phosphatase or Ins(1,4,5)P_3 5-phosphatase activities while Ins(1,4,5)P_3 3-kinase was inhibited by 50% (Table 2).

1 was also tested in a couple of functional models. In the rat parotid slice assay based on the methods of WATSON and DOWNES[10] and BERRIDGE *et al.*[11], **1** gave a dramatic increase in IP1 level ($EC_{50} \sim 1$ mM). However, the carbachol-stimulation was abolished even at low concentration of **1** (Fig. 4).

In the guinea pig trachea model[12], isolated trachea was pretreated with indomethacin (5 μM). The addition of 0.3 mM **1** produced short-lived contraction of the trachea, and in the presence of **1** the response to carbachol was not well maintained; the rate of offset of the remaining response to carbachol, upon the addition of atropine, after a necessarily brief period of stimulation was not significantly affected (Fig. 5). It should be noted that long periods of exposure to agonist, up to 2 hours, are required for full expression of the response to LiCl. Consistent with the observations in the biochemical model, **1** is also not Li^+-like in these two functional models.

Discussion

The identification of the phosphoinositide cycle as a likely target for lithium action arose from the work of SHERMAN and colleagues[13], who demonstrated a profound elevation of inositol-1-phosphate and a corresponding decrease in free inositol in the brains of rats treated systemically with lithium. This was attributed to inhibition of *myo*-inositol mono-phosphatase. The development of potent and specific inhibitors of inositol mono-phosphatase could lead to novel drugs for the treatment of mania and depression. The mode of inhibition of **1** for inositol mono-phosphatase with respect to both substrate and Mg^{2+} are non-competitive and thus is not Li^+-like. However it demonstrates substantial selectivity for the mono-phosphatase, among other phosphatases in the phosphoinositide cycle, and is more potent than lithium ($IC_{50} = 1$ mM). This selectivity may be exploited.

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