Inhibitors of *myo*-Inositol Monophosphatase, ATCC 20928 Factors A and C

Isolation, Physico-chemical Characterization and Biological Properties

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During the course of a screening program for inhibitors of *myo*-inositol monophosphatase we fermented the strain ATCC 20928, a known producer of L-671,776. We now show that this strain produces a complex of at least three sesquiterpenic compounds, L-671,776 (termed factor B) and two structurally related substances, termed factors A and C. Both factors A and C, like L-671,776, exhibited inhibitory activity against *myo*-inositol monophosphatase. Six other fungi producing the above mentioned compounds were also isolated and taxonomically characterized.

myo-Inositol monophosphatase (IMPase) is a key enzyme in the phosphoinosotide (PI) cell signaling pathway. In recent years, this enzyme has attracted a lot of attention as it has been suggested to constitute the mechanism by which lithium exerts its therapeutic effects in manic depression. However, lithium has several adverse effects and consequently several groups have attempted to find new inhibitors of this enzyme which may exhibit an improved safety profile compared with lithium.

To date only one inhibitor, L-671,776, has been isolated from natural sources. This compound, isolated from a fungal strain, is a non-competitive inhibitor of IMPase and belongs to the sesquiterpenic class of molecules. Sesquiterpenic molecules have also been described to inhibit other targets *e.g.*, complement factors, HIV protease, *etc.*[1,2].

We devised a high volume assay to detect IMPase inhibitors in microbial fermentation broths[4].

During this search program, the fungus ATCC 20928, described as *Memnoniella echinata*, producing the known IMPase inhibitor named L-671,776[1], was fermented to obtain this compound as a reference standard. In our fermentation conditions, we obtained a mixture of three active factors, two of these have not been previously described as inhibitors of IMPase. We also isolated six fungal strains which produced inhibitory activities against IMPase and all strains are members of *Stachybotrys/Memnoniella* genera.

Materials and Methods

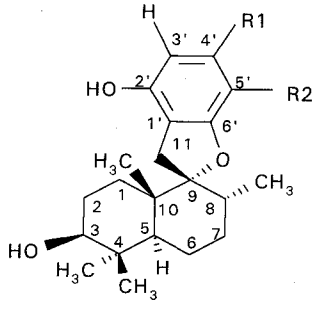
Producing Organisms, Morphological, Cultural and Physiological Characteristics

The producing organisms were recovered from soil samples collected from geographically varied regions. Morphological observation of the strains was made using phase-contrast microscopy after growth on various media at 24°C for 10 days.

Detailed observation of cultural characteristics was performed by light microscope. Microscopic preparation was undertaken as described in Ainsworth's Dictionary of the fungi. Cultural characteristics were determined according to the methods of DOMSCH *et al.*[9].

Color assignments were made using the "A Dictionary of Color" by MAERZ and REA PAUL[7].

Fig. 1. Chemical structure of ATCC 20928 A, B and reduced factors.



	R1	R2
Factor A (**1**)	CHO	CHO
Factor B (**2**)	CH_2OH	CHO
Reduced factor (**3**)	CH_2OH	CH_2OH

Culture Media and Fermentation Condition

A 2 ml portion of frozen culture ATCC 20928 in glycerol was transferred to three slants containing solid medium PCA (mashed potato 20 g/liter, mashed carrot 20 g/liter, agar 20 g/liter). Approximately 10 days later, a slant was used to inoculate a 500 ml baffled Erlenmeyer flask containing 100 ml of sterile medium (pH 6.8) containing atomized corn steep (5 g/liter), starch potato (10 g/liter), glucose (10 g/liter), and a mixture of trace elements (1 ml). The mixture of trace elements contained $FeSO_4 \cdot 7H_2O$ (1 g/liter), $MnSO_4 \cdot 4H_2O$ (1 g/liter), $CuCl_2 \cdot 2H_2O$ (25 mg/liter), $CaCl_2 \cdot 2H_2O$ (100 mg/liter), H_3BO_3 (56 mg/liter), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (19 mg/liter) and $ZnSO_4 \cdot 7H_2O$ (200 mg/liter).

The flask was incubated at 25°C and 150 rpm, for 3 days and was used to inoculate a 15 liter fermenter. The fermentation was carried out at 25°C under a 6 liters/minute range of airflow and agitation rate at 350 rpm for about 4 days.

IMPase Inhibitory Assay

The time course of production of inhibitory activities and the purification of each factor was followed using the IMPase assay as described by VINCENDON *et al.*[4].

Chromatographic Analyses

TLC analyses were done on HPTLC silica gel plates (Merck, Silica gel 60 F254) using acetone-hexane 4:6 (v/v) as mobile phase. Under these conditions, factors A, B and C show a Rf of 0.48, 0.26 and 0.23 respectively. HPLC analysis were carried out on a HP-1090 Liquid Chromatograph apparatus, using a Beckman ODS column, 5 μ (4.6 × 240 mm); mobile phase A: aqueous $HCOONH_4$ (2.5 g/liter)-CH_3CN 8:2; mobile phase B: aqueous $HCOONH_4$ (2.5 g/liter)-CH_3CN 2:8; gradient from 40% to 70% of phase B in 30 minutes; flow: 1 ml/minute; UV detection at 254 nm. Under these chromatographic conditions, the retention time of the three factors A, B and C was 20.9 minutes, 14.4 minutes and 9.3 minutes respectively.

Reduction of Factors A and B with $NaBH_4$

3 mg of pure factor A were dissolved in 1 ml of MeOH together with 2 mg of $NaBH_4$. The solution was stirred at room temperature and the progression of the reaction followed by TLC analysis. After 1 hour the reaction mixture was concentrated under reduced pressure, and the powder obtained was transferred on the top of a silica gel SPE column (Supelclean extraction tubes LC-Si, 1 ml) previously conditioned with acetone. The column was washed with the same solvent to remove the more lipophilic unreacted substrate, and then was eluted with methanol to recover the reduced compound (**3**). The solvent was removed under vacuum and the resulting product analyzed by FAB-MS. The same procedure was followed for the reduction of factor B. The two reduced compounds show the same Rf on TLC silica gel plates (0.12), and mass spectrum of both samples shows a major peak at 373 ($MH^+ - H_2O$).

Spectroscopical Analyses

UV spectra were recorded on a Shimadzu UV-visible spectrophotometer UV-160A. Positive ion FAB and Chemical Ionization (PICI) mass spectra were obtained on a Finnigan TSQ 700 triple stage quadrupole mass spectrometer. For the positive FAB experiments a saddle field atom gun was used with Xe gas at 8 kV voltage and 0.23 mA current. The PICI spectra were carried out with isobutane as reagent, ion source temperature 120°C, electron energy 120 eV and gas line pressure 4600 mTorr. The NMR spectra were recorded using a Bruker AMX-600 spectrometer.

Results

Production and Isolation of Factors A, B, and C

ATCC 20928 was fermented in a 15l jar fermenter (see materials and methods). The time course of production of the inhibitory activity against IMPase was determined at various times by assaying the S112-resin elutes (Table 1, see materials and methods). The production of factor A was also detected by direct injection of the fermented broth in the HPLC system (see materials and methods). As shown in Table 1 little or no activity was detected before 48 hours and maximal activity was achieved between 96~120 hours.

For purification purposes the strain was therefore fermented for 120 hours under these conditions and eight liters of fermented broth were harvested and the mycelium was removed by filtration with Hyflo filter matrix. Since the mycelium contains only the known inhibitor L-671,776[1], factor B (**2**) the cell cake was discarded. The remaining IMPase inhibiting complex was adsorbed from the filtrate (3 hours stirring, batch-wise) onto 250 ml of S112 polystyrene resin (The Dow Chemical Company). The resin was then recovered, washed with water and eluted with 1 liter of EtOH. The eluate was concentrated under reduced pressure and the aqueous residue lyophilized to yield 8.2 g of crude ATCC 20928 complex.

Table 1. Time course of production of IMPase inhibitory molecules during fermentation in KF medium.

Time course (hours)	pH	Wet cell mass (mm)	Inhibitory activity (%)
48	6.6	15	50
72	6.6	17	70
96	6.8	20	85

7.7 g of this crude preparation was suspended in acetone, and the insoluble material was removed by centrifugation. The supernatant was concentrated under reduced pressure and the oily material (3 g) was then applied to the top of a silica gel column (particle size 230~400 mesh ASTM, Merck 9385), previously equilibrated in hexane. The column chromatography was performed on a medium pressure apparatus (Buchi Preparative LC-system B680-A) and eluted with hexane containing increasing amounts of acetone (linear gradient from 0% to 40% of acetone in 120 minutes).

Three different IMPase inhibiting fractions, namely factors A (**1**), B (**2**) and C, were isolated from the column, brought to dryness, redissolved in *t*-butanol and lyophilized, yielding 265, 498 and 78 mg of pure factors A, B and C, respectively.

Physico-chemical Properties and Structure Elucidation of Factors A, B and C

The physico-chemical properties of the three factors are summarized in Table 2. Both factors A and B gave a single peak by HPLC reverse phase analysis and single spots by TLC analysis. Factor C showed two unresolved bands.

The UV spectra of the three factors were slightly different and showed shifted absorption band depending on the pH of the solution. The UV spectrum of factor A exhibited significant maxima at 247, 308 and 356 nm in $CHCl_3$ solvent. Factor B showed maxima at 230, 286 and 330 nm in MeOH solvent; in basic conditions it showed maxima at 248, 290 and 337 nm. Factor C exhibited significant absorption maxima at 282 and 337 nm in basic conditions.

All three compounds were very soluble in methanol, DMSO, acetonitrile and chloroform.

Table 2. Physico-chemical properties of ATCC 20928 complex.

	Factor A (**1**)	Factor B (**2**)	Factor C
Appearance	White powder	White powder	White powder
Molecular formula	$C_{23}H_{30}O_5$	$C_{23}H_{32}O_5$	$C_{23}H_{30}O_6$
FAB-MS	387 (MH^+)	389(MH^+)	403 (MH^+)
UV (organic)	247, 308, 356 nm[b]	230, 286, 330 nm[c]	—
UV (KOH 0.1 N)	—	248, 290, 337 nm	282, 337 nm
HPLC[a]	Rt: 12.5	Rt: 8.7	
TLC[a]	Rf: 0.48	Rf: 0.26	Rf: 0.23

[a] Method reported in General section.
[b] Solvent: $CHCl_3$.
[c] Solvent: MeOH.

Factors A, B, and C were analyzed by mass spectrometry under Fast Atom Bombardment condition (FAB-MS). Factor C was also analyzed by Chemical Ionization (PICI). The FAB-MS spectra of all three factors showed peaks corresponding to the quasi molecular ion $[MH]^+$ at *m/z* 387 (factor A), at *m/z* 389 (factor B) and at *m/z* 403 (factor C), respectively. It is possible to determine the molecular weight of these compounds only if the FAB-MS experiments are carried out using thioglycerol as matrix, otherwise, for all three factors, the peak corresponding to the loss of water was the major peak. The complete ^{13}C and ^1H NMR signal assignment for factor A is reported in Table 3.

The molecular formula of factor A was determined, by comparison between NMR and Mass spectra, as $C_{23}H_{30}O_5$, and corresponds to the oxidized form of factor B at the position 4'. A further confirmation of the similarity of these two molecules can be obtained by reduction of (**1**) and (**2**) with $NaBH_4$ in methanol. In fact, they gave the same reaction product corresponding to the diol (**3**).

A comparison of the spectroscopic data with those

Table 3. ^{13}C and ^1H NMR data (δ cm^{-1}) in CD_3CN for ATCC 20928-A.

Position	δ_H	δ_C	HMBC correlation[a]
1	1.03, 1.62	24.86	15
2	1.4~1.7; 1.9~2	25.83	
3	3.29	75.51	13, 14
4	—	38.32	13, 14
5	2.25	40.96	13, 14, 15
6	1.56	21.65	7
7	1.54	31.88	12
8	1.9~2.0	37.73	11, 12
9	—	101.65	11
10	—	43.16	11, 15
11	2.87 d (17.5) 3.20 d (17.5)	31.45	8, 9, 10, 1', 2', 6'
12	0.75	15.79	7, 8
13	0.87	22.67	3, 4, 5, 14
14	0.96	28.81	3, 4, 5, 13
15	1.04	16.37	1, 5, 10
1'	—	120.08	11
2'	—	158.50	11, 3'
3'	6.80	109.12	3'
4'	—	139.00	7',8'
5'	—	112.15	8', 3'
6'	—	168.00	11
7'	10.52	193.3	3'
8'	10.34	188.94	7'

[a] Correlated carbon number.
The NMR spectra were recorded on a Bruker AMX 600 spectrometer in CD_3CN solution at 303K For the HMBC spectrum a total of 128 transients were averaged for each of the 512 increments in t_1, and 2048 complex points in t_2. The ^1H-^1H COSY spectra were obtained in the phase sensitive double quantum filter mode.

reported in literature, showed that factor A is similar to Mer-NF5003 F[2]. The UV, MS and NMR spectra of factor B were in complete agreement with data reported in the literature, for L-671,776[1,2]. During our analytical studies, a reassignment of the structure of this inhibitor was also done[3]. Comparison of UV, MS and preliminary NMR data of factor C with that reported in the literature suggests that factor C was a new more oxidized sesquiterpenic compound.

IMPase Inhibitory Activity

The inhibition of IMPase enzyme by factors A, B, and C was also determined[5,6]. The enzyme reaction was performed either in the absence or in the presence of various concentrations of either factor A, B, or C, to determine the dose-response curves. The IC_{50} (the amount of compound which inhibits the enzyme activity by 50%) of the factors is shown in Table 4. To further determine if the aldehyde moiety on the aromatic ring was important for enzyme inhibition we also tested reduced factors. In fact the reduction of the aldehyde to an alcoholic function resulted in a significant (**2**) or total loss of activity (**3**). Similarly, the more oxidized product, factor C, also showed a reduced activity.

Taxonomy

Six soil fungi producing IMPase inhibitory activities were isolated. All of these strains exhibit essential and diagnostic features of the genus Stachybotrys. This genus is very similar to genus Memnoniella as described by JONG and DAVIS[8] and DOMSCH *et al.*[9]. A summary of the significant morphological features of the strains is included in the following description.

Table 4. IC_{50} for inhibition of IMPase by factor A, B, and C.

	IC_{50} (μM)
Factor A (**1**)	70
Factor B (**2**)	460
Reduced factor (**3**)	>2500
Factor C	200

Colonies on PCA agar effuse, black, velvety with stroma and setae absent. Mycelium superficial composed of septate, branched flaxes hyphae; hyphae hyaline to olivaceous-brown or black. Conidiophores macronematous, unbranched. Conidiogenous cell monophialidic, in groups at the end at the apex of each stipe or branch, clavate with a very small opening and no collarette. Conidia ellispsoidal, smooth, blackish brown to black when mature.

The cultural characteristics of the strain ATCC 20928 and the strains on various agar media are shown in Table 5.

Effects of Fermentation Media on the Production of Factors A, B and C.

Fermentations of four of the producing strains were carried out in two different media. A loopful of spores of each producing strain was inoculated into a 500-ml Erlermeyer flask containing either 100 ml of KF or 100 ml of Memno medium[1]. The fermentations were carried out with continuous shaking at 200 rpm and 25°C.

The time course of production was followed by aseptically removing aliquots (5 ml) of the culture filtrate and, after binding to S-112 resin, elution with ethanol. Little or no IMPase inhibitory activity was produced in the Memno medium for up to 120 hours. By contrast, all strains produced IMPase inhibitory activity by about 96 hours of fermentation in KF medium (Fig. 2). The eluted extracts were also subjected to TLC and compared with reference standards. TLC fractionation showed that strain GE 61392 while producing factor A and C did not produce any significant amount of factor B in the medium. On the other hand, strain GE64665 which produced both factor A and B did not produce factor C. The ratio of the factors A, B, and C also varied depending on the producing strains (Fig. 3).

Discussion

ATCC 20928 has been previously reported to produce L-671,776. Our results show that this fungus produces a complex of at least three closely related sesquiterpenic

Table 5. Cultural characteristics of ATCC 20928 and strains isolated from natural product screening.

Medium	Growth	Aerial mycelium	Reverse side	Exudates
Malt extract agar (MEA)	Good	Abundant, white	Yellowish	None
Czapek yeast autolysate (CYA)	Good	Abundant, white	Yellowish	None
Malt czapek agar (MCZ)	Good	Abundant, white~black	Black	Black

Fig. 2. Time course of production of IMPase inhibitory activity by four isolated strains.

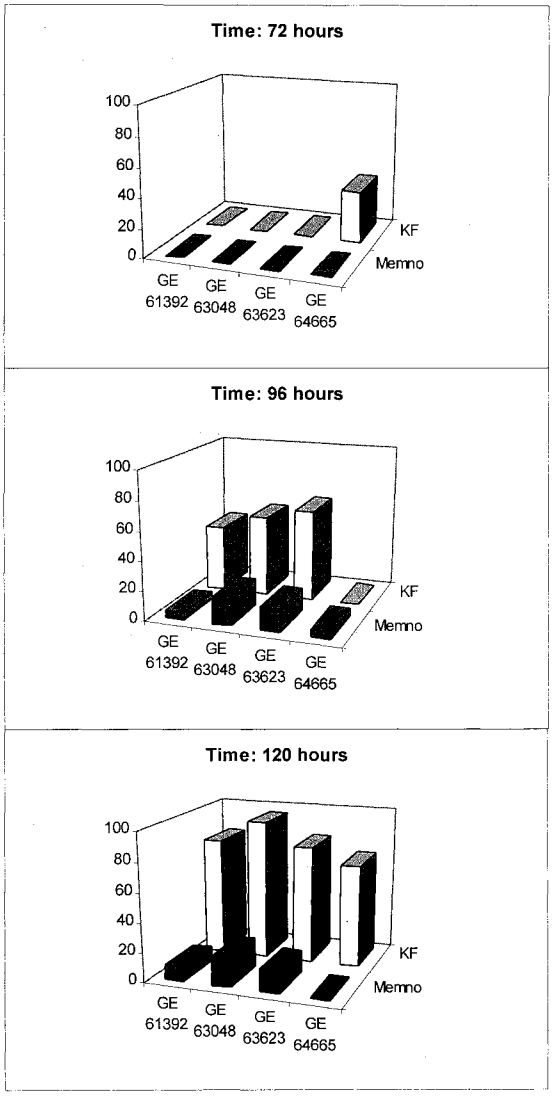
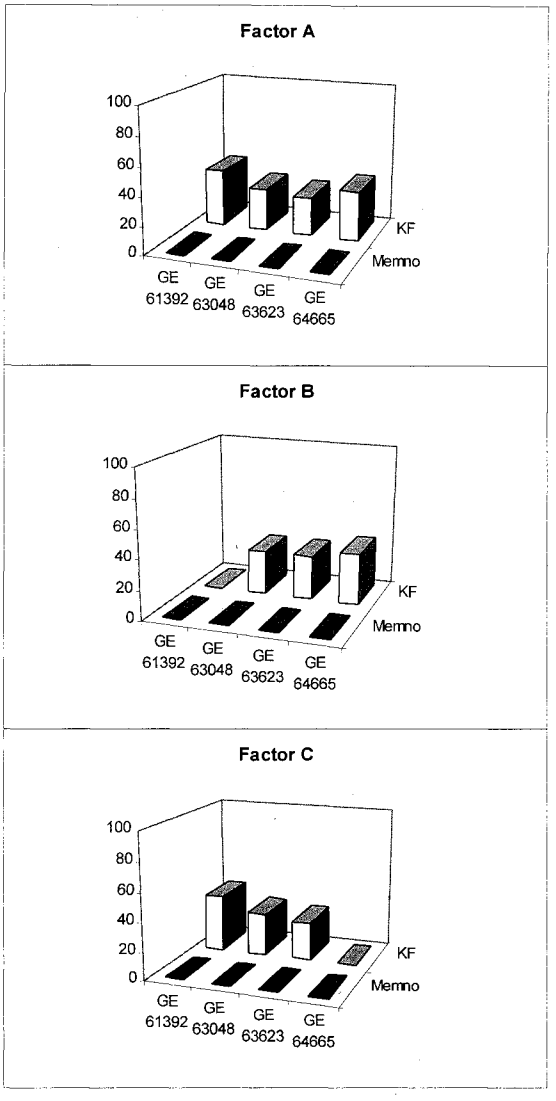


Fig. 3. Production of the three factors A, B and C by four isolated strains in two different media.



compounds. All three compounds, termed factors A, B, and C, inhibited IMPase. A comparison of the IC_{50} of the three factors, however, shows that factors A and C are more potent inhibitors of IMPase compared with the previously identified L-671,776: IC_{50} of 0.07 mM, 0.45 mM, and 0.2 mM for factors A, B, and C respectively. The mass of the three factors has been determined and the structure of factors A and B has also been elucidated by NMR. The three factors differ by the oxidation degree of the functional groups on the aromatic ring. The presence of the aldehydic moiety is important for the inhibitory activity against the IMPase enzyme, in particular reduction of the aldehydic functions to the alcohol results in complete loss of inhibition.

Six different strains producing IMPase inhibitory activities were isolated and all belong to the Stachybotris/Memnomiella genera. Although all strains produced the three factors, the time course of production and the total amount of each factor produced varied between different strains. We are currently undertaking more detailed studies to understand these differences.

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