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# Isolation and Characterisation of a Prenylated *p*-Terphenyl Metabolite of *Aspergillus candidus* Possessing Potent and Selective Cytotoxic Activity; Studies on Mechanism of Action

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We describe the discovery and properties of a prenylated *p*-terphenyl metabolite of the fungus *Aspergillus candidus*. The compound (**1**) possesses potent cytotoxic activity against a range of tumour and other hyper-proliferative cell lines. Cell cycle analysis shows that in mouse keratinocyte (BALB/MK) cells treated with **1**, the cell cycle is arrested in early S phase, indicative of an antimetabolite effect. Furthermore, cellular cytotoxicity can be reversed by addition of exogenous pyrimidine but not purine nucleosides to the cell culture medium. It is therefore likely that compound **1** selectively inhibits pyrimidine biosynthesis, and it is this property which accounts for its potent cytotoxic properties.

*p*-Terphenyl metabolites are rare in nature. The first to be described was terphenyllin[1], isolated from *Aspergillus candidus* in 1975 (see Figure 1). Subsequently 4″-deoxy, 3-hydroxy and 3,3″-dihydroxy analogues have been described, all of them isolated from strains of *Aspergillus candidus*[2~4]. Terferol (3′-demethyl-4,4″-dideoxyterphenyllin), an inhibitor of cAMP-dependent phosphodiesterase, has been identified in cultures of *Streptomyces showdoensis* by NAKAGAWA *et al.*[5]

Very recently KAMIGAUCHI *et al.*[6~8] have described the terprenins, prenylated analogues of terphenyllin which possess potent immunosuppressant activity. In this paper we report the identification of a prenylated *p*-terphenyl metabolite (**1**, Figure 1) which we show to possess potent cytotoxic activity against BALB/MK and other hyperproliferative cell lines. The compound appears to be identical to 4″-deoxyterprenin[6].

## Materials and Methods

### Fermentation

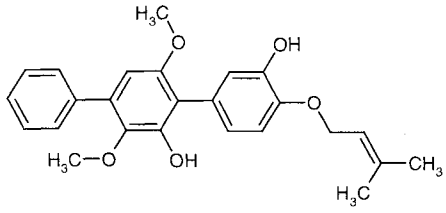
The producing organism, *Aspergillus candidus* Link F23967, was isolated from possum dung collected in Northland, New Zealand. The organism was inoculated from agar plugs stored in water into a 250 ml Erlenmeyer flask containing 50 ml of medium which comprised peptone 1%, malt extract 2.1%, glycerol 4%, Junlon 110 (Honeywell & Stein) 1%. The culture was incubated at 25°C for 10 days on a rotary shaker operated at 250 rpm with a 50 mm diameter orbital motion. 3% (v/v) portions of the developed inoculum were used to inoculate further 250 ml Erlenmyer flasks (50 ml medium) or 2,000 ml Florence flasks (400 ml medium) containing the same medium and these were incubated as described above.

Two hundred ml of bulked shake flask developed inoculum was used to inoculate a 20 litre fermenter containing 10 litres of production medium. Eight hundred ml of bulked Florence flask developed inoculum was used to inoculate a 70 litre fermenter containing 50 litres of production medium. The production medium consisted of glucose 0.2%, tomato paste 4%, oatflour 1.5%, polypropylene 2000 (K & K Greef) 0.05%. The fermentations were controlled to a temperature of 25°C. The cultures were agitated at 1000 rpm (20 litre vessel) or at 750 rpm (70 litre vessel) and aerated at 0.5 VVM.

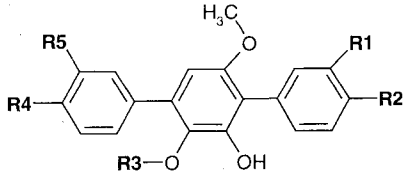
### Isolation of **1**

After 7 days fermentation, the cultures were combined and cells were removed from the fermentation broth by vacuum filtration through Dicalite 478. Dicalite (1%)

Fig. 1. Structures of terphenyls



Compound **1**



Generic structure (see key below)

| R1 | R2 | R3 | R4 | R5 | Name |
|---|---|---|---|---|---|
| $OCH_3$ | $OCH_2CH=C(CH_3)_2$ | Me | OH | H | 3-Methoxyterprenin |
| OH | $OCH_2CH=C(CH_3)_2$ | Me | OH | H | Terprenin |
| H | OH | Me | OH | H | Terphenyllin |
| H | OH | Me | H | H | 4′-Deoxyterphenyllin |
| OH | OH | Me | OH | H | 3-Hydroxyterphenyllin |
| H | H | H | H | H | Terferol |
| OH | OH | Me | OH | OH | 3,3″-Dihydroxyterphenyllin |

was added to the broth as filter aid. The cell mass (*ca.* 3 kg) was stirred with acetone (10 litres) for 30 minutes, then re-filtered through the original filter bed and the filtrate taken. The cell mass was stirred again with acetone (10 litres) for 30 minutes and refiltered. The filtrates (*ca.* 21 litres) were combined and concentrated (Buchi R175 rotary evaporator, bath temperature 50°C) to remove organic solvent. Saturated sodium chloride solution (1.5 litres) was added to the concentrate (1.5 litres) and this was extracted with hexane : dichloromethane 1 : 1 (2 × 1 litre).

The organic layer was taken and reduced to an oil, which was dissolved in dichloromethane : hexane (1 : 1, 600 ml) and a further 200 ml hexane was added. This was applied to a silica column (Merck Kieselgel 60, 5 cm × 50 cm, prepared in hexane) and developed with dichloromethane : hexane 1 : 1. The fraction eluting between 3 litres and 6 litres was taken and evaporated to a red oil. This was extracted with acetonitrile (100 ml). The red tar remaining was discarded and the acetonitrile extract was diluted to 1.4 litres with 60% (v/v) acetonitrile/water containing 0.1% (v/v) formic acid, which was filtered to remove insoluble red tar. The filtrate was passed through reverse phase silica cartridges (C18 Bond-Elut; Varian Ltd., 10 g size, 350 ml per cartridge). The cartridges were washed with 60% (v/v) acetonitrile/water containing 0.1% (v/v) formic acid (150 ml per cartridge). The combined effluents and washings were combined and evaporated to remove acetonitrile. The product precipitated as pale pink waxy plates, and these were recovered by filtration. After washing with 40% (v/v) acetonitrile/water containing 0.1% (v/v) formic acid, the crude product was redissolved in hot acetonitrile (50 ml) then diluting the solution to a volume of 800 ml with 40% (v/v) acetonitrile/water. This was further diluted with hot water (100 ml) whereupon the solution became cloudy. After storage at 4°C for 24 hours, the product was recovered by filtration, washed with 40% (v/v) acetonitrile/water, then dried *in vacuo* over $P_2O_5$ to yield **1** (2.71 g) as colourless needles.

## Conditions for NMR/MS Data Acquisition

NMR spectra were measured on a Bruker AMX500 spectrometer using standard pulse sequences. MS spectra were acquired on a Finnigan Mat LCQ mass spectrometer under electrospray positive ion mode

(ESI+ve) conditions.

### MK Cell Culture

For proliferation and cell cycle studies, the Balb/MK cell line, (referred to as 'MK' in this manuscript), was used. This is a clonal Balb/c mouse epidermal keratinocyte cell line (WEISSMAN and AARONSON[9]). The cells were routinely grown in calcium-free S-MEM (minimum essential medium) supplemented with 8% FCS, Glutamax™, antibiotic/antimycotic solution, and 4 ng/ml epidermal growth factor (EGF, Sigma). For studies to assess effects of exogenously added purines and pyrimidines, MEM Alpha was used. This medium contains 10 mg/ml ribonucleosides (adenosine, cytidine, guanosine and uridine) and deoxyribonucleosides (2'-deoxyadenosine, 2'-deoxycytidine HCl, 2'-deoxyguanosine and 2'-deoxythymidine). Proliferation was also measured in standard S-MEM in the presence of 10 $\mu$M uridine, cytidine, adenosine, guanosine or thymidine (Sigma), alone or combined. All tissue culture reagents were purchased from Gibco BRL (Life Technologies) unless otherwise stated.

### Cell Proliferation Assays

Cells were harvested using trypsin/EDTA, and plated at $5 \times 10^3$ cells/well in 96 well microtitre plates, in 100 $\mu$l of medium. The next day, **1** was diluted in the appropriate growth medium, at double the final required concentration, from a 10 mM stock solution formulated in DMSO, and added at 100 $\mu$l per well. Medium alone was added to control wells. Cells were incubated at 37°C, 5% $CO_2$ for 4 days. Medium was removed by aspiration, and cell biomass was estimated by staining cells by addition of 100 $\mu$l methylene blue (Sigma, 0.5% in 50:50 ethanol: water), and incubation at room temperature for at least 30 minutes. Stain was washed off with water, the plates were allowed to dry, and stain was released from the cells by the addition 100 $\mu$l of solubilisation solution (1% *N*-lauroyl sarcosine, sodium salt, in PBS; Sigma). Plates were read on an Anthos plate reader, measuring the optical density (OD) at 620 nm. Percent inhibition of cell growth was calculated relative to non-compound treated control wells.

### Cell Cycle Analysis

For cell cycle analysis, MK mouse keratinocyte cells were cultured in 6 well plates at $3 \times 10^5$ cells/well, in the presence of 4 ng/ml EGF. **1** was added at 10, 1, 0.1, 0.01 or 0 $\mu$M for 3 days. Cells cultured in the absence of EGF were also included as a control. Cells and medium were then harvested and cell nuclei were stained with propidium iodide using a CycleTEST PLUS Reagent Kit (Becton Dickinson), and samples were analysed using a fluorescence activated cell sorter (FACScan, Becton Dickinson), reading at 488 nm. DNA profiles were then analysed using CellFit software.

## Results and Discussion

### Structure Determination

The structure of **1** was determined by a full analysis of both 1D and 2D NMR together with MS data. MS data showed an ion at *m/z* 407, consistent with that for the protonated molecular ion $(M+H)^+$ of **1**. Subsequent comparison with spectral data published for 4''-deoxyterprenin[6] confirmed that the two compounds were indeed identical.

### *In Vitro* Cytotoxic Activity

**1** was evaluated against a range of tumour and other hyperproliferative cell lines. The compound exhibited potent activity against many of the cell lines evaluated (Table 1), and in addition showed much weaker activity against control cell lines (data not shown). In view of the apparently selective cytotoxic activity displayed by **1**, cell cycle analysis was undertaken to try to elucidate the mechanism of action of the compound.

### Cell Cycle Analysis

Mouse keratinocyte (MK) cells were analysed for their cell cycle distribution (Table 2 and Figure 2). The data showed that in the absence of EGF the cells arrested in $Go/G_1$ (Figure 2A). In the presence of EGF, a profile typical of proliferating cells was seen, with around 50% of the cell population within the S-phase fraction (Figure 2B). **1**, at concentrations less than 10 $\mu$M, caused an apparent accumulation of cells in S-phase (Figure 2D, E and F), suggestive of an anti-metabolite mode of action. In the presence of 10 $\mu$M **1**, the cells were effectively arrested in $Go/G_1$, with a profile identical to that of the cells grown in the absence of EGF (Figure 2C).

### Cell Proliferation Assays; Effect of Added Nucleosides

**1** showed potent inhibiton of proliferation of the MK cells, with an $IC_{50}$ of 14 nM (Figure 3). In view of the S-phase specific cell cycle effects, the effect of added nucleosides on activity of **1** was assessed by making cell and compound dilutions in either MEM Alpha

Table 1. *In vitro* cytotoxic activity of compound **1** against selected tumour and other hyperproliferative cell lines.

| Cell line | Description | $IC_{50}$ ($\mu$M) |
|---|---|---|
| HB4a | Immortalised normal breast epithelial cell line | 0.185 |
| HB4a/R4.2 | HB4a transfected with mutant Ras | 0.171 |
| HB4a/C5.2 | HB4a transfected with c-erbB2 (overexpressed) | 0.065 |
| MDAMB468 | EGFR overexpressing breast carcinoma | 0.477 |
| HN5 | EGFR overexpressing head and neck carcinoma | 0.064 |
| A431 | EGFR overexpressing vulval carcinoma | 0.055 |
| MK | Mouse keratinocytes (dependent on EGF for growth) | 0.014 |
| BT474 | c-erbB2 overexpressing breast carcinoma | 1.121 |
| NCI-N87 | c-erbB2 overexpressing gastric carcinoma | 0.105 |
| CALU3 | c-erbB2 overexpressing lung carcinoma | 3.368 |
| NR6/N12A | NR6 expressing mutant (activated) c-erbB2 | 0.868 |
| MCF7 | Oestrogen receptor +ve breast carcinoma | 0.133 |
| T47D | Breast carcinoma | 0.272 |
| A549 | Non small cell lung carcinoma | 0.247 |
| HT29 | Colon carcinoma | 3.687 |
| SW620 | Colon carcinoma | 0.610 |
| RKO | Colon carcinoma | 0.810 |
| LoVo | Colon carcinoma | 0.046 |
| Du145 | Prostate carcinoma | 0.539 |

Table 2. Cell cycle analysis of MK cells.

| Treatment | | Cell cycle distribution (% of cycling cells) | | |
|---|---|---|---|---|
| EGF | Compound **1** ($\mu$M) | $Go/G_1$ | *S* | $G_2/M$ |
| − | 0 | 95.4 | 4.2 | 0.4 |
| + | 0 | 39.8 | 52.5 | 7.7 |
| + | 10 | 87.9 | 9.3 | 2.7 |
| + | 1 | 67.7 | 30.3 | 2 |
| + | 0.1 | 14.2 | 77.5 | 8.3 |
| + | 0.01 | 27.2 | 61.5 | 11.3 |

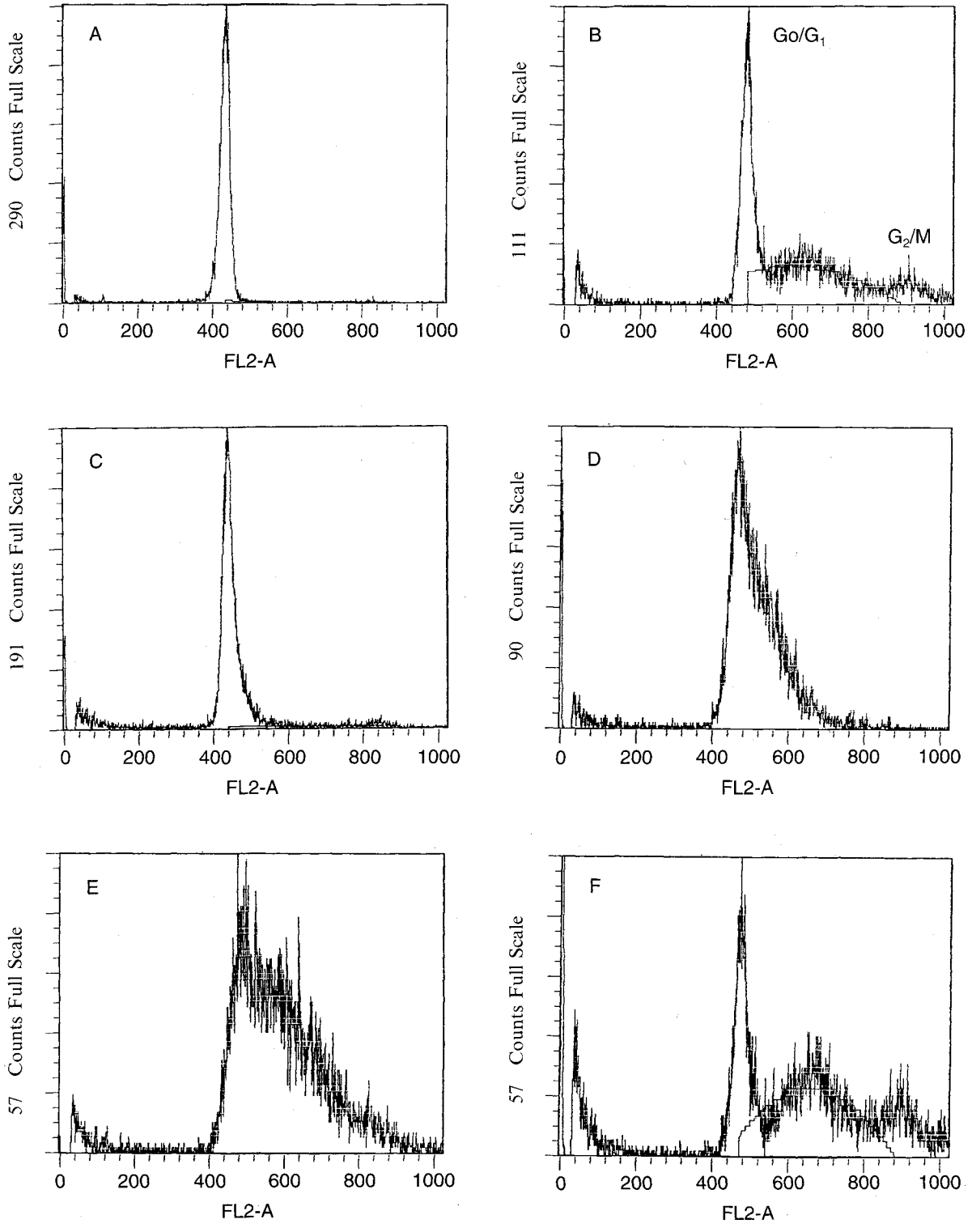
Cells were stained with propidium iodide after 3 days incubation with **1**. The values for the percentage of cycling cells in each particular phase of the cell cycle are quantitation of the flow cytometry experiments shown as histograms in Figure 2.

medium, or standard S-MEM containing 10 $\mu$M adenosine, guanosine, cytidine or uridine added individually, or in combination. In MEM Alpha or with exogenously added pyrimidines the anti-proliferative activity of **1** was partially reversed. In MEM Alpha, the $IC_{50}$ for the MK cells increased from 14 nM to 316 nM (Figure 3). In S-MEM, the $IC_{50}$ was increased from 44 nM to 2.6 $\mu$M in the presence of the pyrimidine nucleosides, uridine and cytidine (Figure 4). In contrast, the addition of the purines, adenosine and guanosine, had no effect on the inhibition of proliferation by **1**, suggesting that this compound could be having its effects at the level of pyrimidine biosynthesis.

The fourth enzyme on the pyrimidine biosynthetic pathway (dihydroorotate dehydrogenase) catalyses the conversion of dihydroorotic acid to orotic acid. It is interesting to note that certain well known inhibitors of this enzyme *e.g.* brequinar[10], show some structural similarity to naturally occurring terphenyls such as **1**. It is possible that inhibition of dihydroorotate dehydrogenase could explain the cytotoxic activity demonstrated by **1**, though this is purely speculative and has not been tested experimentally.

We have also evaluated terphenyllin and related non-prenylated analogues for whole cell cytoxicity, and none of them showed potency comparable to **1** (unpublished data). The compound was also evaluated against the National Cancer Institute's panel of human tumour cell lines. The compound inhibited the proliferation of many of these with low nanomolar $IC_{50}$ values; an analysis of this data will form the basis of a further publication (manuscript in preparation).

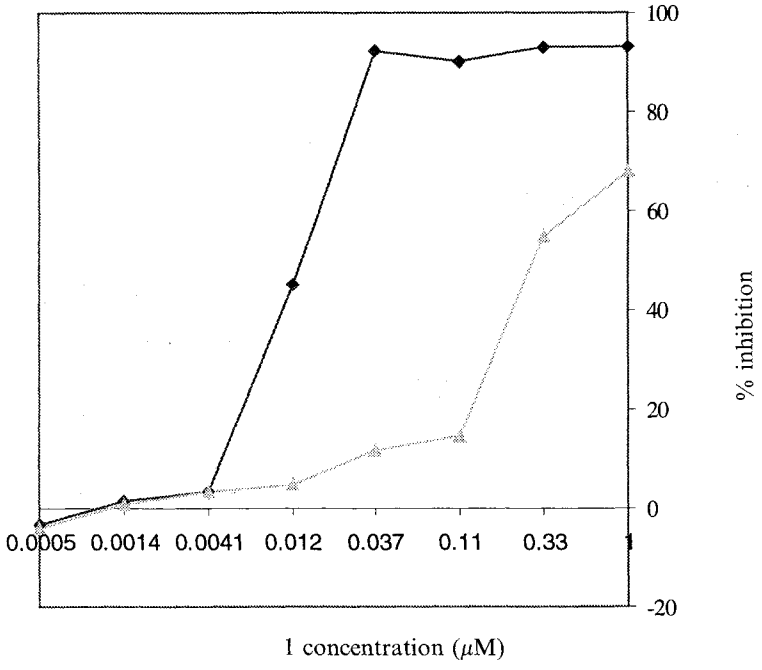
Fig. 2. Effects of **1** on MK cell cycle.




MK cells were exposed to control medium containing 0.1% DMSO, in the absence of EGF (A), control medium containing 0.1% DMSO, in the presence of EGF (B), **1** (in the presence of EGF) at 10 $\mu$M (C), 1 $\mu$M (D), 0.1 $\mu$M (E), or 0.01 $\mu$M (F), for 3 days. DNA content was assessed by propidium iodide uptake as described in 'Materials and Methods'. Ten thousand cells were analysed per determination, and cell cycle distribution was analysed using CellFit software. See Table 2 for quantitation of the $Go/G_1$, S and $G_2/M$ peaks.

Fig. 3. Potent Inhibition of MK cell proliferation by **1**, and rescue in Alpha MEM.

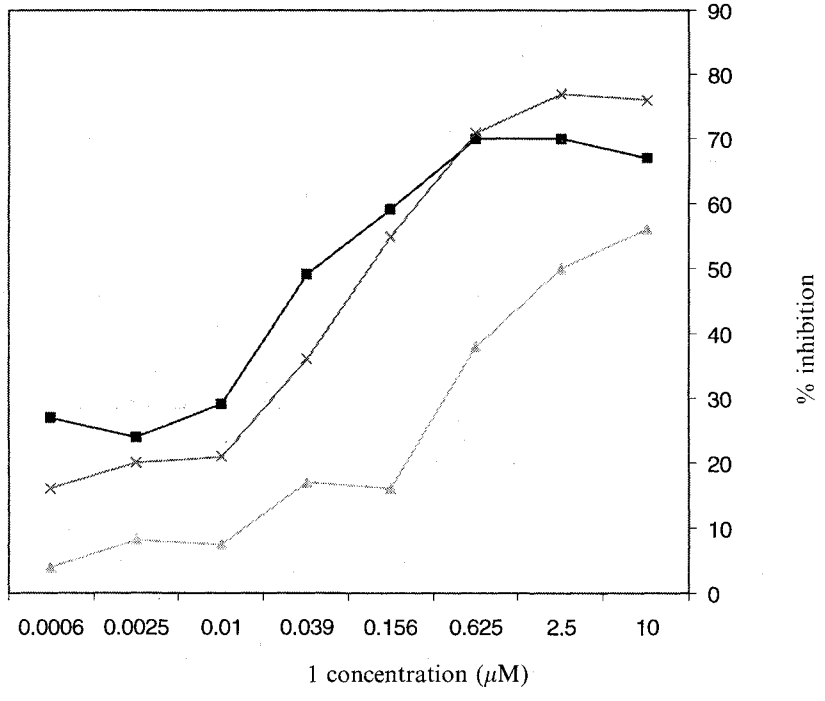
◆ S-MEM, ▲ Alpha-MEM.




MK cells were incubated for 4 days with **1** diluted in either standard growth medium (S-MEM), or Alpha MEM, which contains 10 mg/ml adenosine, cytidine, guanosine and uridine. Proliferation was measured as described in the 'Materials and Methods'.

Fig. 4. Antagonism of MK cell cytotoxicity induced by **1** by pyrimidine, but not purine, nucleosides.

■ S-MEM, ▲ uridine/cytidine, × adenosine/guanosine.




MK cells were incubated for 4 days with **1** diluted in S-MEM containing either 10 μM of the pyrimidines uridine and cytidine, or 10 μM of the purines adenosine and guanosine. Proliferation was measured as described in the 'Materials and Methods'.

## Conclusion

We have shown, through *in vitro* studies, that compound **1** (a *p*-terphenyl metabolite of *Aspergillus candidus*) is likely to be an inhibitor of pyrimidine biosynthesis. This activity probably accounts for the observed cellular cytoxocity of the compound. It is intriguing that the presence of an oxygen-linked isoprene substituent has such a dramatic effect on cytotoxic potency. Although the molecular basis for this activity has not yet been fully elucidated, it could potentially be ascribed to inhibition of a pyrimidine biosynthetic enzyme.

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