

## Production of Antitumor Antibiotic GKK1032B by *Penicillium citrinum*, an Endophytic Fungus Isolated from *Garcinia mangostana* Fruits

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### Abstract

Endophytic fungi are considered as a good source to produce important secondary metabolites with interesting bioactivities. In a continuation of our studies towards the search for environmentally friendly bioactive compounds from Sri Lankan flora we investigated the secondary metabolites produced by the endophytic fungi *Penicillium citrinum* isolated from the fruits of *Garcinia mangostana*. The pure culture of the *P. citrinum* was grown on potato dextrose broth (PDB) media. After four weeks fermentation, fungal medium and mycelium were extracted with ethyl acetate. Chromatographic separation of the EtOAc extracts over silica gel, Sephadex LH-20 and PTLC furnished a peptide-polyketide hybrid compound, GKK1032B and citrinin. GKK1032B has been reported previously from an unidentified species of *Penicillium* as an antitumor antibiotic. The present work suggested that the unidentified species could be *Penicillium citrinum*.

**Keywords:** GKK1032B; Citrinin; *Penicillium citrinum*; *Garcinia mangostana*; Endophytic fungi

### Introduction

Endophytes are typically fungi and bacteria that inhabit plants without causing disease symptoms [1]. Some endophytes have been shown to benefit their hosts by improving nutrient availability, overcoming abiotic stress and as biocontrol agents against plant pathogens [2]. Almost all classes of vascular plants examined to date are found to host endophytic organisms [3]. Recently, increasing attention has been paid to the production of bioactive compounds by endophytic fungi associated with plants. In the previous papers, we reported the isolation of naphthopyrones from *Aspergillus* sp. isolated from *Limonia acidissima* [4] and phenazine derivatives from *Nigrospora oryzae*, isolated from *Coccinia grandis* [5]. In a continuation of our studies on bioactive secondary metabolites produced by fungal endophytes associated with Sri Lankan plants, we investigated metabolites of an endophytic fungus isolated from the fruits of *Garcinia mangostana* (mangostin). Various parts of the *G. mangostana* is used to treat for various diseases in traditional medicine, especially pericarp to treat for trauma, skin infections, abdominal pain, dysentery and wounds [6]. *G. mangostana* have very diverse bioactivities including antiallergy, antibacterial, antifungal, anti-inflammatory, antioxidant, antitumoral, antiviral, cytotoxic, immunomodulatory and neuroprotective properties [7-11]. Over 68 xanthone-type constituents have been reported from *G. mangostana* [12]. *P. citrinum* is a commonly occurring filamentous fungus with a worldwide distribution and this species has been isolated from various substrates such as soil, (tropical) cereals, spices and indoor environments [13]. GKK1032B is a unique peptide-polyketide hybrid compound which has been reported previously from an unidentified species of *Penicillium* as an antitumor antibiotics [14,15]. In this paper we report the identification of the fungus, which produced GKK1032B as *Penicillium citrinum*.

### Materials and Methods

#### General

A VWR ultrasound cleaner, USC 1700D, was used for extraction. Analytical TLC was performed on Merck Kieselgel 60F<sub>254</sub> aluminum plates. TLC spots were visualized under UV 254 nm and spraying with anisaldehyde agent followed by heating. Column chromatography (CC) was performed on silica Merck Art No. 7734 or 9385 and gel chromatography was on Sephadex LH-20 Fluka Art No. 20100. <sup>1</sup>H NMR

and <sup>13</sup>C NMR were recorded on a Bruker DRX500 (500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C) spectrometer in CDCl<sub>3</sub> solution. FAB/MS (positive ion mode) spectra were obtained on a JEOL JMS-700 spectrometer with NBA as matrix (Figures 1 and 2).

#### Isolation and identification of the endophytic fungus

Fruits of *G. mangostana* were collected from Central Province of Sri Lanka in April 2014. Fruit was washed with running tap water to remove surface dust and debris. After triple sterilization of fruits with ethanol, 2.5% NaOCl and distilled water, a segment of the inside of the pericarp which touch the fleshy part of the fruit was placed on a potato dextrose agar (PDA) media in a petri-dish (90 mm) and incubated at room temperature. Emerging fungi were isolated after 5 days. To obtain a pure culture of endophytic fungus with grayish green colored upper surface with a velvety texture was serially transferred into PDA media. The fungus was identified as *Penicillium citrinum* through molecular means using internal transcribed spacer (ITS) region of rDNA gene. The results from the BLAST search indicated that the sequence was 100% identical to that of *Penicillium citrinum* (GenBank Accession No. KP013076.1). PCR and DNA sequencing was done by the GeneTech Institute, Sri Lanka. Photographic evidence of the fruits of *G. mangostana* and fungal strain (IFS/MQ-EFME-4/2014) is deposited at the Institute of Fundamental Studies.

#### Extraction and isolation of compounds

Large scale culturing of the fungus was carried out by inoculating *P. citrinum* culture grown on PDA medium to 1 L conical flasks (x 20) containing 400 mL of PDB medium, which were allowed to stand still at room temperature for 10 days, and then incubated while shaking every other day on a laboratory shaker. The medium was filtered after four weeks

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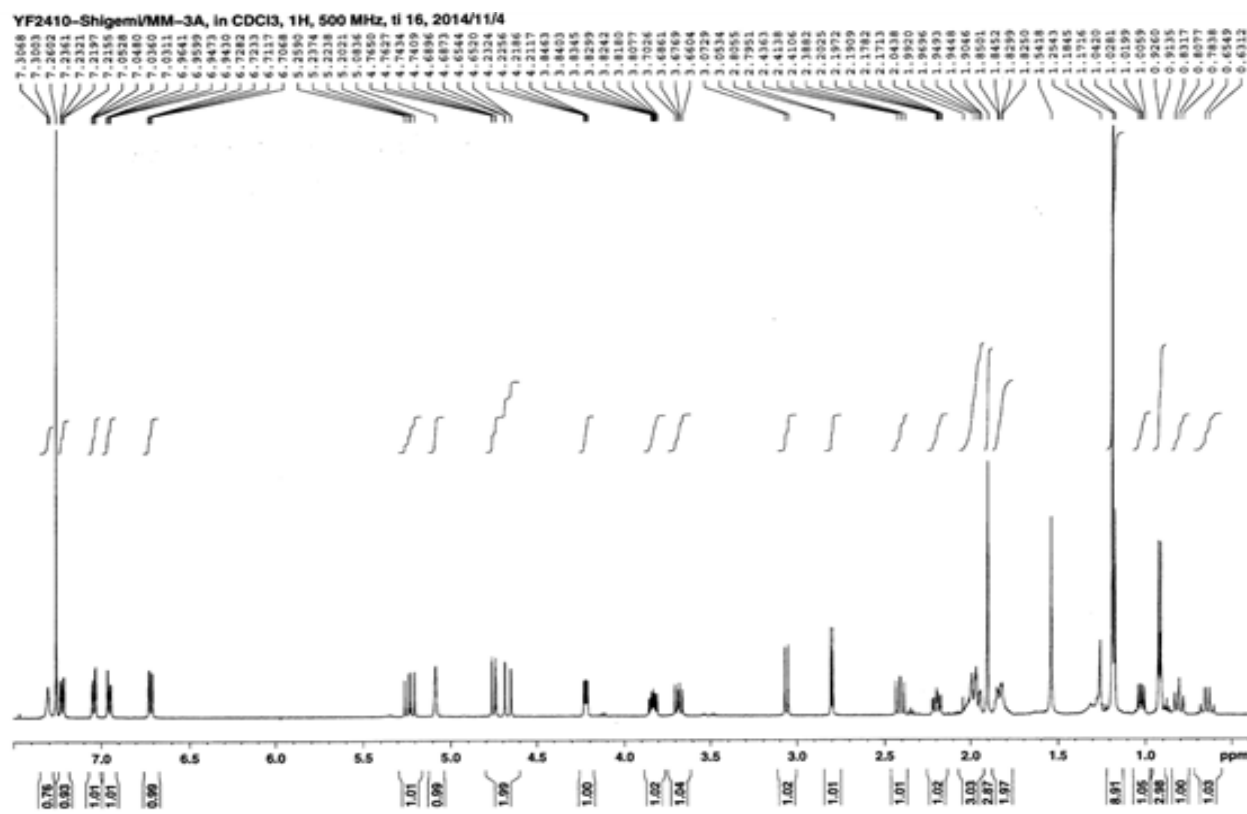


Figure 1: <sup>1</sup>H NMR spectrum (500 MHz, CDCl<sub>3</sub>) of compound 1.

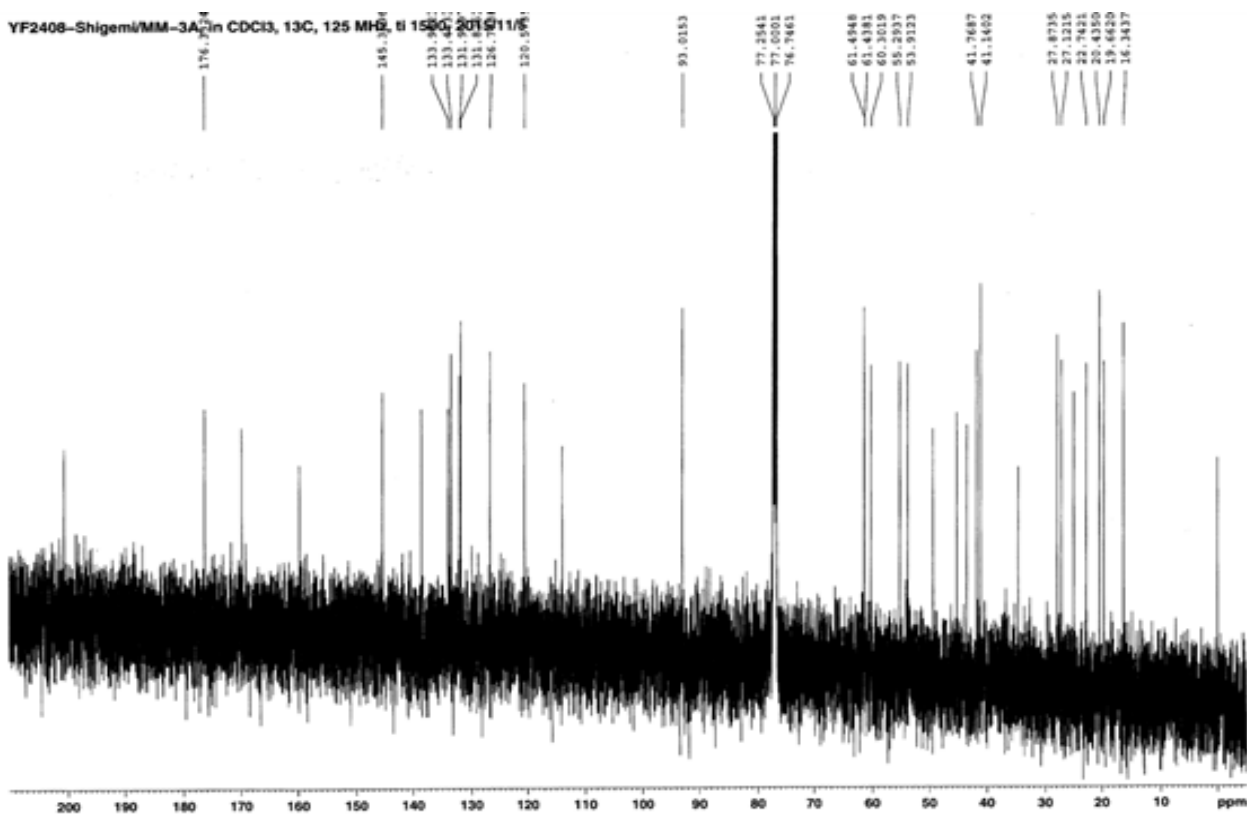


Figure 2: <sup>13</sup>C NMR spectrum (125 MHz, CDCl<sub>3</sub>) of compound 1.

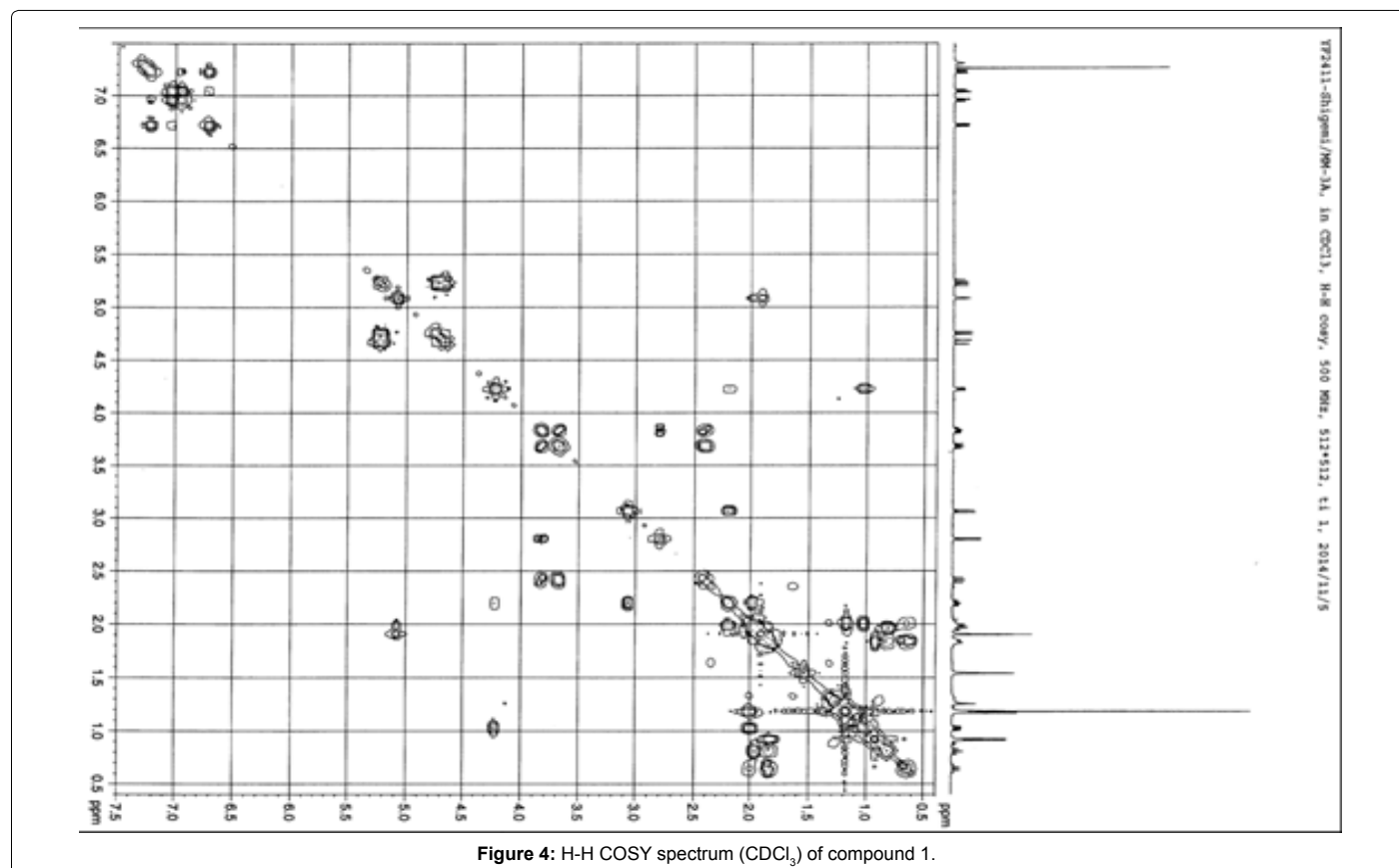
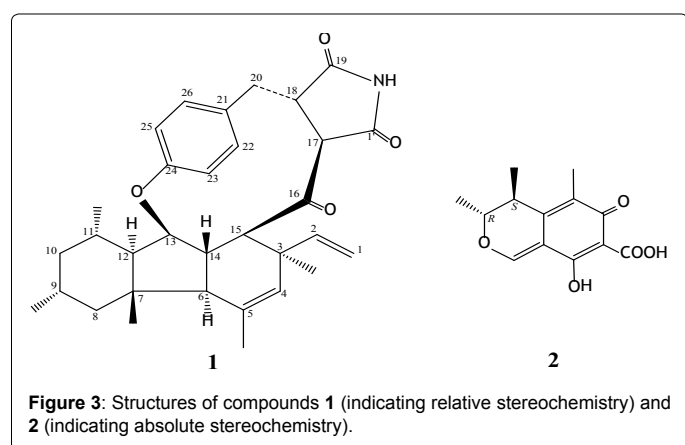
and the filtrate was partitioned three times with EtOAc. Concentration of the EtOAc layer on a rotary evaporator furnished EtOAc extract (2.64 g). The residual mycelium was crushed and extracted with EtOAc to give EtOAc extract (214 mg). TLC analysis indicated that the EtOAc extract from the PDB medium exhibit the same pattern of spots as that from the mycelium. Hence, the two EtOAc extracts were combined and subjected to CC over silica gel, Sephadex LH-20, and preparative TLC to furnished compounds **1** (32 mg), **2** (2.30 g) and ergosterol (100 mg).

**GKK1032B:** Amorphous solid,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz): 4.67 (1H, *dd*,  $J=16.6, 1.1$  Hz,  $\text{H}_a-1$ ), 4.75 (1H, *dd*,  $J=10.9, 1.1$  Hz,  $\text{H}_b-1$ ), 5.23 (1H, *dd*,  $J=16.6, 10.9$  Hz, H-2), 5.08 (1H, *brs*, H-4), 1.98 (1H, *brd*,  $J=11.4$  Hz, H-6), 0.81 (1H, *dd*,  $J=12.0, 12.0$  Hz,  $\text{H}_a-8$ ), 1.95 (1H, *dd*,  $J=12.0, 2.2$  Hz,  $\text{H}_b-8$ ), 1.83 (1H, *m*, H-9), 0.64 (1H, *ddd*,  $J=11.9, 11.9, 11.9$  Hz,  $\text{H}_a-10$ ), 1.82 (1H,

*brd*,  $J=11.9$  Hz,  $\text{H}_b-10$ ), 1.98 (1H, *m*, H-11), 1.02 (1H, *dd*,  $J=11.2, 6.9$  Hz, H-12), 4.22 (1H, *dd*,  $J=6.9, 3.4$  Hz, H-13), 2.19 (1H, *ddd*,  $J=11.4, 9.8, 3.4$  Hz, H-14), 3.06 (1H, *d*,  $J=9.8$  Hz, H-15), 2.80 (1H, *d*,  $J=5.2$  Hz, H-17), 3.83 (1H, *ddd*,  $J=11.2, 8.3, 5.2$  Hz, H-18), 2.41 (1H, *dd*,  $J=12.8, 11.2$  Hz,  $\text{H}_a-20$ ), 3.68 (1H, *dd*,  $J=12.8, 8.3$  Hz,  $\text{H}_b-20$ ), 7.23 (1H, *dd*,  $J=8.3, 2.1$  Hz, H-22), 6.72 (1H, *dd*,  $J=8.3, 2.5$  Hz, H-23), 7.04 (1H, *dd*,  $J=8.4, 2.5$  Hz, H-25), 6.95 (1H, *dd*,  $J=8.4, 2.1$  Hz, H-26), 1.18 (3H, *s*, 3-Me), 1.90 (3H, *s*, 5-Me), 1.18 (3H, *s*, 7-Me), 0.92 (3H, *d*,  $J=6.2$  Hz, 9-Me), 1.18 (3H, *d*,  $J=6.5$  Hz, 11-Me);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 125 MHz): 114.0 (C-1), 145.4 (C-2), 41.8 (C-3), 131.8 (C-4), 138.5 (C-5), 53.9 (C-6), 41.1 (C-7), 49.4 (C-8), 27.9 (C-9), 45.2 (C-10), 27.1 (C-11), 61.5 (C-12 or C-15), 93.0 (C-13), 55.3 (C-14), 61.4 (C-15 or C-12), 200.1 (C-16), 60.3 (C-17), 43.5 (C-18), 176.3 (C-19), 34.7 (C-20), 133.9 (C-21), 131.9 (C-22), 126.7 (C-23), 159.7 (C-24), 120.6 (C-25), 133.4 (C-26), 169.8 (C-1'), 19.7 (C-3-Me), 20.4 (C-5-Me), 24.9 (C-7-Me), 22.7 (C-9-Me), 16.3 (C-11-Me); FABMS (+):  $m/z$  502  $[\text{M}+\text{H}]^+$ .

## Results and Discussion

An endophytic fungus isolated from the surface of the pericarp which touches the fleshy edible fruit part of *G. mangostana* was identified as *Penicillium citrinum* through molecular means using ITS region of rDNA gene. Inoculation of *P. citrinum* culture to PDB, fermentation for four weeks and extraction of the broth and mycelium with EtOAc gave the respective extracts, which were found to be similar by TLC analysis. Chromatographic separation of the combined EtOAc extract over silica, Sephadex LH-20 and final purification by PTLC furnished compound **1**, **2** (Figure 3) and ergosterol. Compound **1** was identified as GKK1032B by detail analysis of  $^1\text{H}$ NMR,  $^{13}\text{C}$ NMR, H-H COSY, HMQC, HMBC, FABMS spectral data and also comparison with the reported data (Figures 4-7). Antitumor antibiotic GKK1032B was first isolated from unidentified



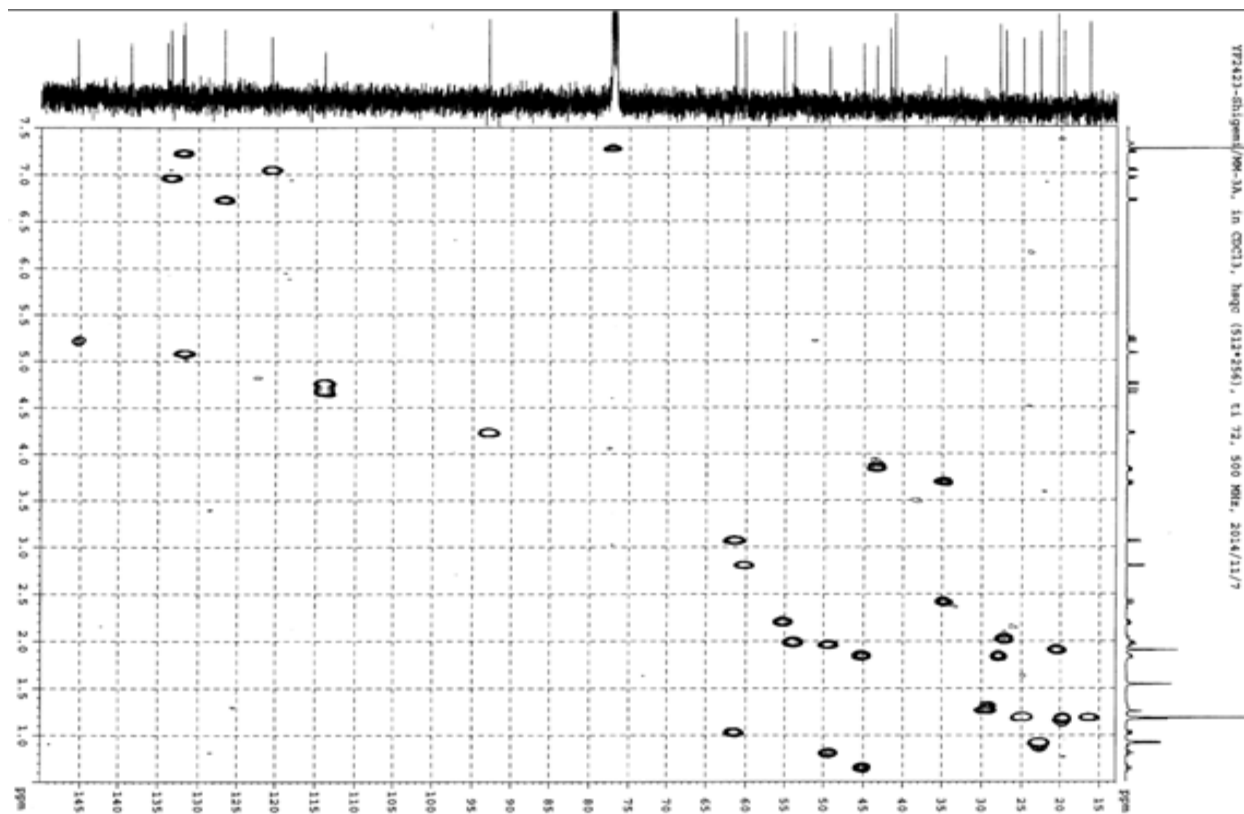


Figure 5: HSQC spectrum ( $\text{CDCl}_3$ ) of compound 1.

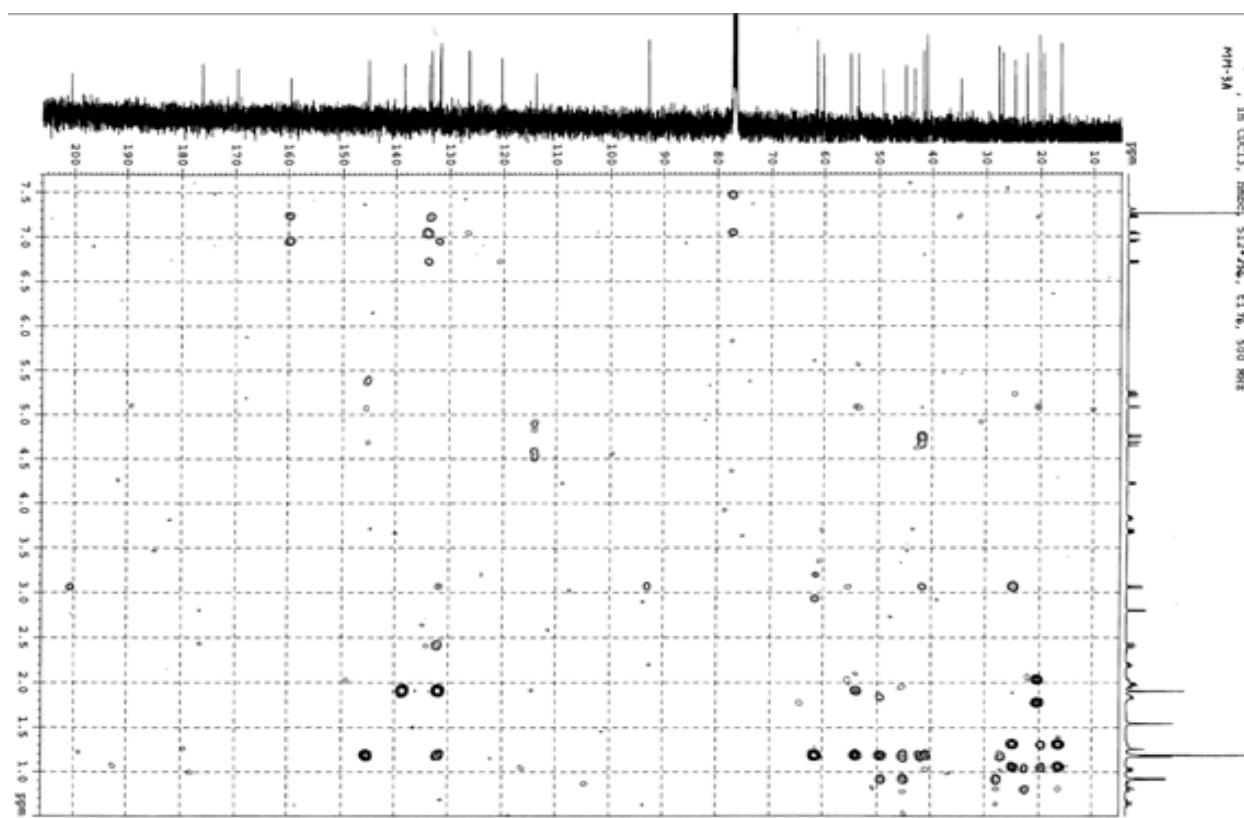
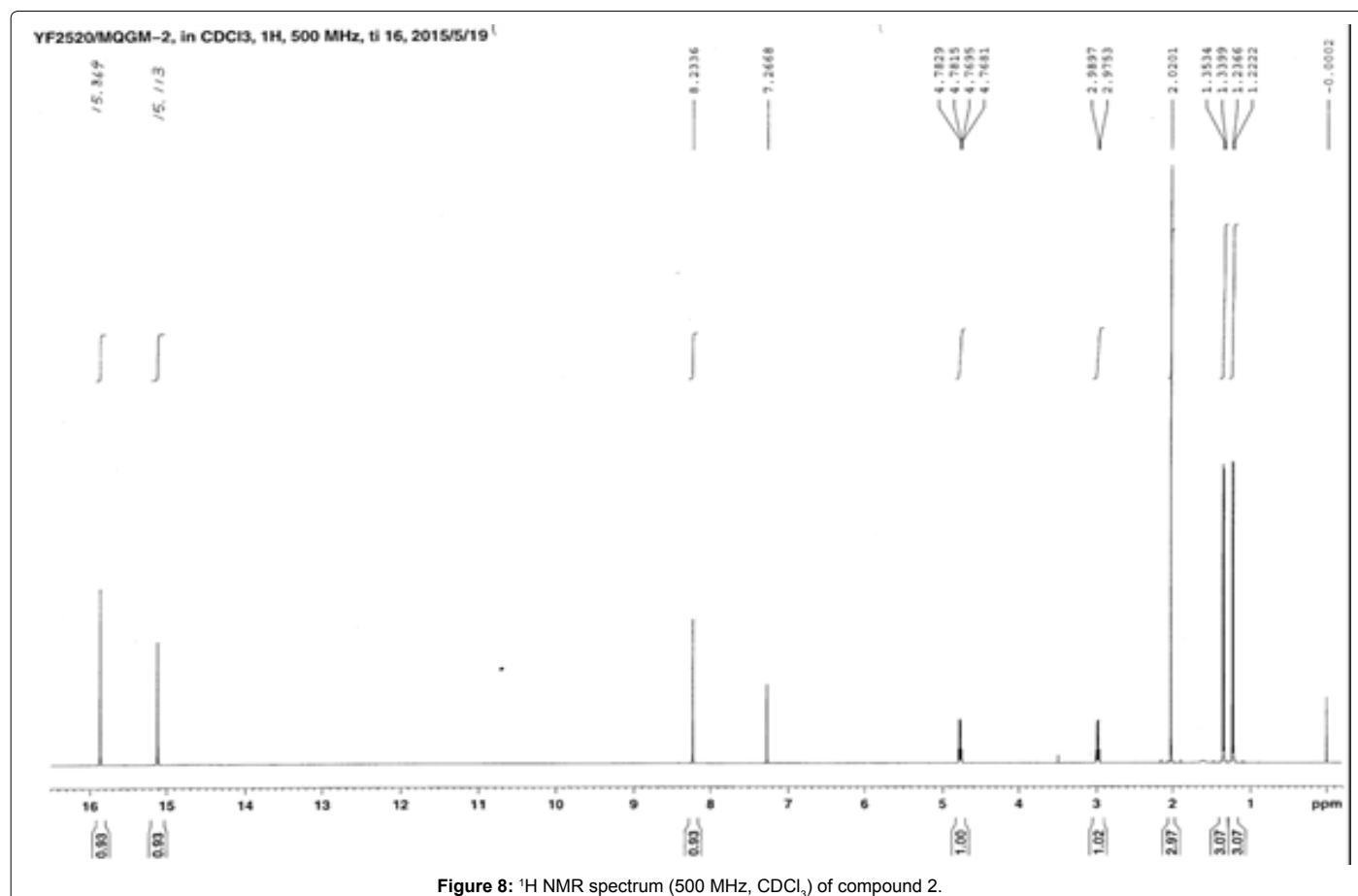
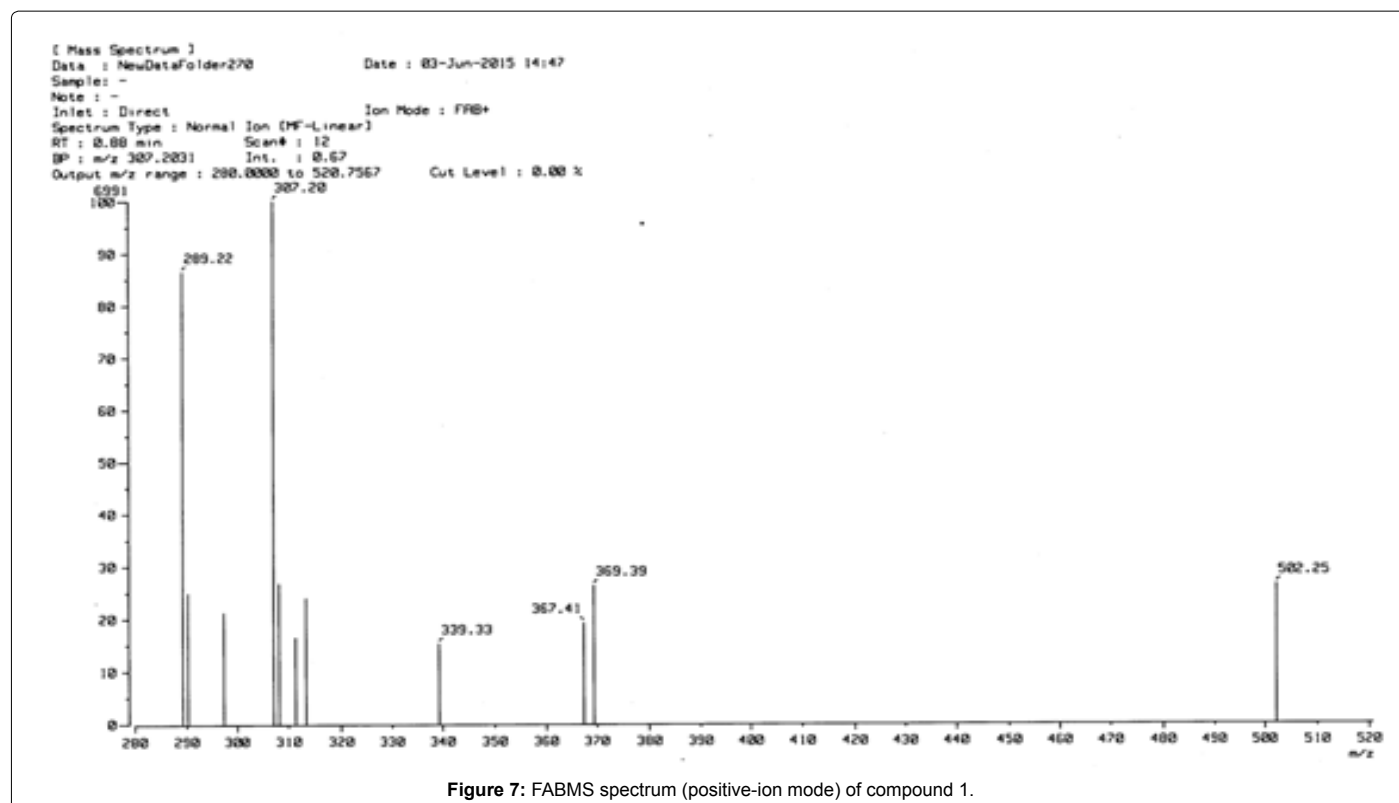
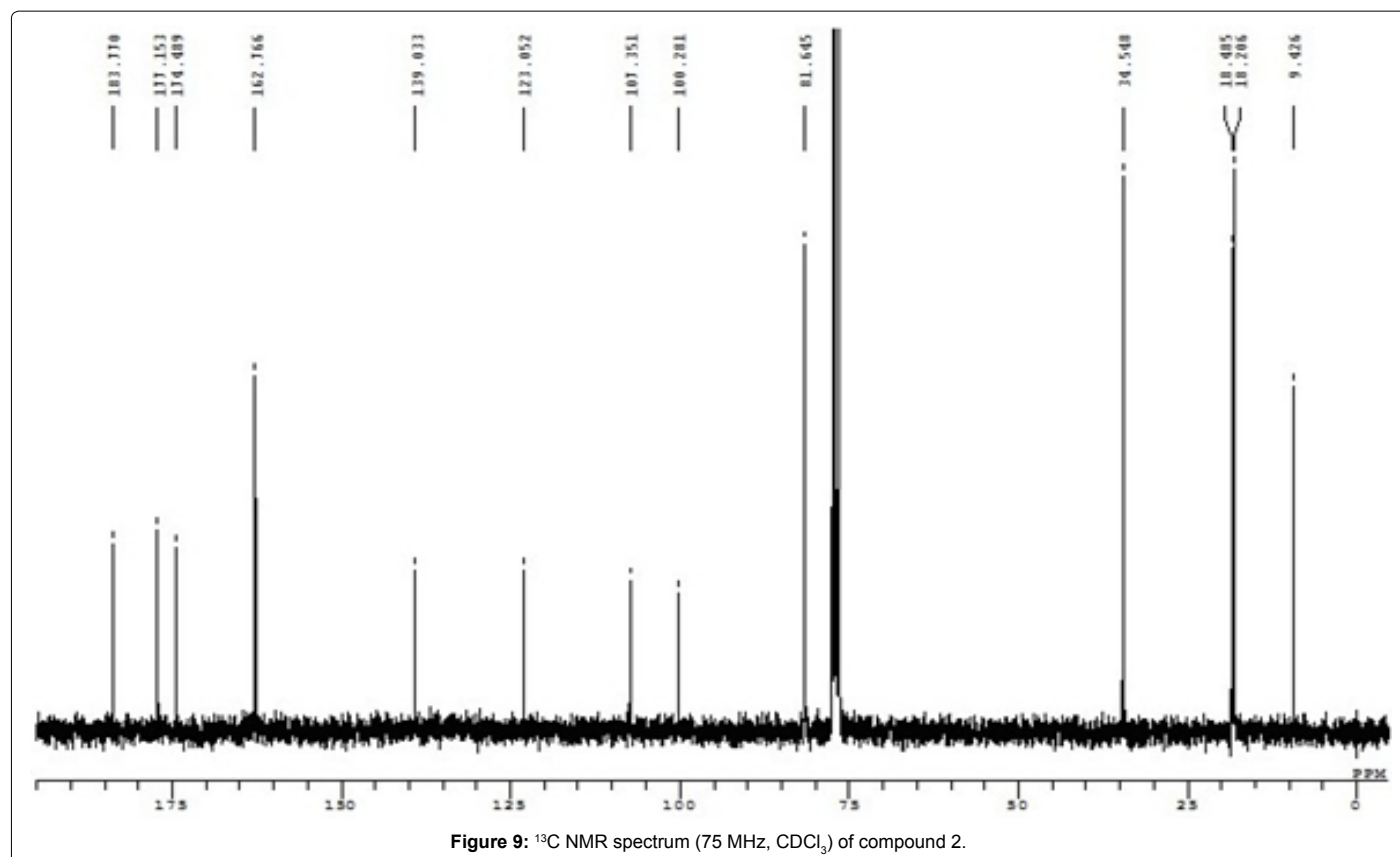


Figure 6: HMBC spectrum ( $\text{CDCl}_3$ ) of compound 1.







**Figure 9:**  $^{13}\text{C}$  NMR spectrum (75 MHz,  $\text{CDCl}_3$ ) of compound 2.

*Penicillium* sp. in 2001 together with GKK1032A<sub>1</sub> and GKK1032A<sub>2</sub> [14,15]. Subsequently GKK1032B has been isolated from an endophytic *Penicillium* sp. isolated from *Melia azedarach* and *Murraya paniculata* [16]. This is the third report of the isolation of GKK1032B. It can be suggested that the two unidentified *Penicillium* strains in the earlier papers are to be *P. citrinum*, since the production of this class of secondary metabolites have not been reported from any other *Penicillium* species thus far. Absolute configuration of 1 remains to be elucidated. Compound 2 was identified as citrinin by comparison of the  $^1\text{H}$ NMR and  $^{13}\text{C}$ NMR spectral data with reported data [17]. Ergosterol was identified based on the  $^1\text{H}$  and  $^{13}\text{C}$ NMR data [18] (Figure 8,9). Citrinin, a nephrotoxin mycotoxin, is consistently produced by *P. citrinum* [19] and Pastre et al. described the isolation of citrinin together with GKK1032B [16]. The isolation of citrinin besides GKK1032B appears to support the validity of the fungal identification. In addition, several other metabolites, tanzowac acid A, quinolactacins, quinocitrinines, asteric acid and compactin are reported to be produced by *P. citrinum* [20].

## Conclusion

The endophytic fungus *Penicillium citrinum* was isolated from *Garcinia mangostana* for the first time. This study established that *P. citrinum* is the producer of the antitumor antibiotics GKK1032B for the first time. It would be worthy of searching for other endophytic fungi from other parts, in particular roots and leaves of *G. mangostana*, since earlier studies had obtained several fungal species, which produced biologically active substances, but without characterization of the fungal secondary metabolites [21,22].

## Supplementary data

1D- and 2D-NMR and FABMS spectra for compound 1 and 1D-NMR spectra for compound 2.

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The authors declare that there are no conflicts of interests.

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