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An Endophytic Fungus from *Nothapodytes foetida* that Produces Camptothecin

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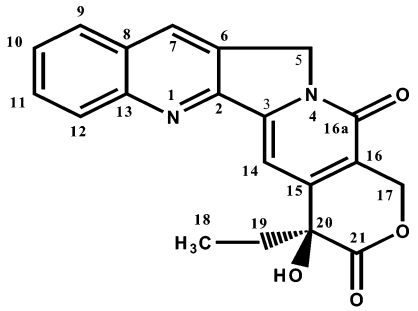
Abstract: A fungal endophytic isolate, camptothecin, has been isolated from the inner bark of the plant *Nothapodytes foetida* from the Western coast of India. The fungus, which belongs to the family Phycomycetes, produced the anticancer drug lead compound camptothecin (**1**) when grown in a synthetic liquid medium (Sabouraud broth) under shake flask and bench scale fermentation conditions. Compound **1** was identified by means of chromatographic and spectroscopic methods. It was also compared with an authentic example for its biological activity against a number of human cancer cell lines. Isolation of an organism producing **1** and its fermentation may, in the future, provide an easily accessible source for the production of this anticancer drug precursor molecule.

Camptothecin (**1**), a pentacyclic quinoline alkaloid, belongs to a group of antineoplastic agents with a unique mechanism of action involving interference with eukaryotic DNA.[1−4] Moreover, one of the primary cellular responses to its exposure is a rapid cessation of RNA synthesis.[5] This alkaloid displays a unique mechanism of action by inhibiting the intranuclear enzyme topoisomerase I, which is required for the swivelling and relaxation of DNA during molecular events, such as DNA replication and transcription.[6]



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20(*S*)-Camptothecin, the naturally occurring enantiomer, was first isolated by Wall et al.[7] from the wood of *Camptotheca acuminata* Decne (Nyssaceae), which is a plant native to mainland China. Camptothecin and its derivatives show strong antineoplastic activity. The drug is already used in China for the treatment of skin diseases.[8] Hycamtin (topotecan) and Camptosar (irinotecan), semisynthetic derivatives of **1**, have been employed clinically for the treatment of ovarian and colon cancers.[9,10] Compound **1** is also used as an insect chemosterilant, a plant regulator, and an inhibitor of the herpes virus.[11] In addition, compound **1** prevents the replication of the influenza virus.[12] Compound **1** and minor camptothecinoids have been obtained in high yield from the Indian tree *Nothapodytes foetida* (Wight) Sleumer (formerly *Mappia foetida*, Icacinaceae),[13−15] commonly known in India as "Kalgur". This small tree is distributed in the western part of peninsular coastal India from Konkan ghats to northern parts of the Kanara, Niligiris, Anamalis, and Pullneys hills. Compound **1** has also been reported to be present in various Japanese plant species, including *N. collina, N. obscura, N. obtusifolia, N. piltosporsides*, and *N. tomentosa*.[16]

Camptothecin (**1**) is not abundant and is only available in relatively low concentrations in the roots of *Nothapodytes* species, which unfortunately demands the uprooting of rare, 50- to 75-year-old trees from the forests. The supplies of **1** available from inconsistent wild sources are, therefore, inadequate when compared to the projected demand. While a synthetic route to **1** has been reported, the yield, after a multistep procedure, is low, commercially insignificant, and

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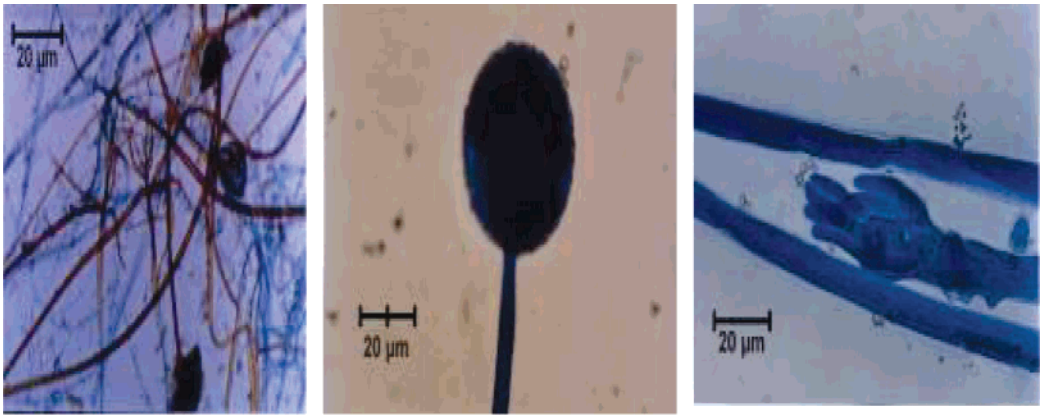

Figure 1. (a) Microscopic view of horizontally growing unbranched stoloniferous hyphae (×500). (b) Microscopic view of the young sporangium of endophytic fungus (×1000). (c) Microscopic view of horizontally growing unbranched stoloniferous hyphae (×1000).

hence nonviable.[17−19] Therefore, it is essential to find alternative sources of **1** to meet the pharmaceutical demand.

Herein we report, for the first time, the production of the quinoline alkaloid camptothecin by an endophytic fungus (RJMEF001) isolated from *N. foetida* (Figure 1a−c), a plant from Konkan ghats in India and presently being maintained in the botanical gardens of Regional Research Laboratory, Jammu. Molecular analysis of the fungus based on a large subunit (LSU) ribosomal RNA gene revealed 99.8% similarity to *Entrophospora infrequens* and also to other related taxa, e.g., *Rhizopus oryzae* strains UWFP 973 and 846 (98.6%). Further investigations of this nature are currently underway. The fungal strain has been deposited at MTCC, Chandigarh, India (MTCC 5124), and a PCT application has also been filed.[20]

A literature survey shows that the antitumor diterpenoid paclitaxel and its congeners have been reported as being produced from an endophytic fungus called *Taxomyces andreanae*.[21−22] There is no published report that **1** might be produced by any microorganism associated with a plant species or growing independently in nature.

The identity of **1** in the fungal isolates was confirmed by chromatographic and analytical methods, such as optical rotation and UV, IR, CD, LC/MS, LC-MS/MS, HRMS, and ^{1}H and ^{13}C NMR spectra. The quantitative estimation of **1** by HPLC against standard **1** (derived from plant) indicated a yield of 18 μg/mg of the chloroform extract after 6−7 days of incubation of the isolated microorganism under shake flask conditions. The electron impact mass spectrum (EIMS) of fungal camptothecin (**1**) was identical (Figure 2) to the published spectrum[23] of this molecule from a plant source, having a molecular ion peak at *m/z* 348 with characteristic fragments at *m/z* 319 (M − ethyl), *m/z* 304 (M − CO_2), *m/z* 291 (*m/z* 319 − CO), *m/z* 290 (*m/z* 319 − CHO), *m/z* 275 (*m/z* 304 − ethyl), *m/z* 248 (*m/z* 275 − HCN), and *m/z* 247 (*m/z* 275 − CO). In the ESIMS, the molecular ion of camptothecin exhibited *m/z* 349 $(M + H)^+$, and in the MS/MS mode, characteristic fragment peaks (Figure 2) could be observed.

The biological activity of fungal **1** was tested using an in vitro cytotoxicity assay[24] against human cancer cell lines (A-549 for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer) in comparison with the standard **1**, resulting in comparable activities.

Optimization of fermentation conditions for the efficient production of **1** by fermentation is underway. This may lead to the development of an economical and eco-friendly process for the production of camptothecin (**1**) by fermentation to meet the ever-increasing demand for the compound as a unique anticancer drug precursor molecule.

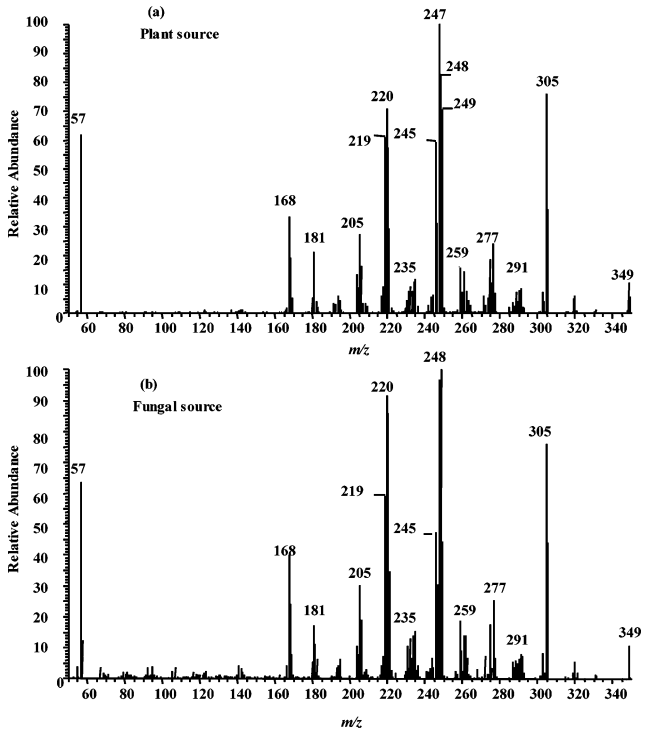

Figure 2. ESIMS/MS of **1** from (a) plant source and (b) fungal source.

Experimental Section

General Experimental Procedures. Low-resolution MS was performed by EI ionization (Finnigan-MAT 8000) at 70 eV, with a direct inlet probe at 252 °C. A Finnigan TSQ 7000 with ESI ionization in the MS/MS mode was used. The optimal collision energy (Figures 2a,b) was determined by means of an ICL procedure controlling the automatic switching between different voltages, with a step size of 0.5 V/scan to 40 V. During this procedure, the analytes were injected via a Rheodyne valve with a 2 μL injection loop at a concentration of 10 μg mL^{-1}. A prescan voltage settling time of 20 min and 0.4 s for one complete cycle (four transitions) was used for selected reaction monitoring (SRM). HRMS was done using a JEOL JMS/SX 102 A FAB ion source (matrix, 3-nitrobenzyl alcohol; calibration, PEG 400; resolution, 10 000) and an Apex III FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) with a 7 T superconducting magnet. Positive ions were

produced in an external Apollo electrospray ion source (Bruker Daltonics, Billerica, MA) with a flow rate of 2 mL min^{-1}. Infrared multiphoton dissociation (IRMPD) and activation of ions in the ICR trap were performed using a CO_2 J48-2 laser with 25 W maximum power output (Synrad, Mukilteo, WA). The optical rotation measurement was performed using a Perkin-Elmer 341 polarimeter with a tube of 10 cm cell path length using $CHCl_3$–MeOH (8:2) as solvent. CD was performed using a JASCO J715 spectropolarimeter, with a Hellma precision quartz glass Suprasil cuvette, a 1 mm light path length, and $CHCl_3$–MeOH (4:1) as solvent. FTIR Bruker IFS (KBr) was used for recording the IR spectra. UV spectra were obtained using a Varian CARY 100 BIO, 1 cm cuvette, and $CHCl_3$–MeOH (4:1) as solvent. For ^1H and ^{13}C NMR spectra a Bruker AMX 600 instrument was used.

Isolation of Endophytic Fungi. Fresh plant material was taken, and small stems explanted from the fully matured *Nothapodyte foetida* tree (containing a measurable concentration of **1**) were treated with 95% ethanol as a disinfectant. Pieces of the inner bark of the stem were placed on aqueous agar (AG) and incubated at 28 ± 2 °C until fungal growth started. The tips of the fungal hyphae were removed from the AG and placed on a rich synthetic mycological medium (e.g., Sabouraud agar, SBA, containing dextrose 4%, peptone 1%, and agar 2%). The pure culture, thus obtained, was preserved by lyophilization, as well as by cryopreservation at −70 °C. The fungus grows as a white cottony mycelium when young. The well-developed mycelium is branched, fast growing, and spreads on the solid medium. Aerial hyphae are produced after growth for 5–7 days and turn black due to sporulation. Microscopic slides were prepared by following standard methods.[25]

Isolation of Total Genomic DNA. Total DNA was isolated from the mycelial mass using the standard method[26] with slight modifications. The DNA was resuspended in a suitable volume of TE buffer (10 mmol of Tris-HCl, pH 8.0, 1 mmol, EDTA). DNA was quantified spectrophotometrically using a Biophotometer (Eppendorf, Hamburg, Germany).

Identification of the Isolate. The fungus was identified using a Microseq D2, large subunit (LSU) rDNA fungal sequencing kit ABI (Applied Biosystems, Foster City, CA). The LSU ribosomal gene (~300 bp) was amplified and sequenced on an ABI Prism 310 genetic analyzer (ABI, Foster City, CA). The DNA sequences thus obtained were submitted to the ribosomal gene database (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov) and the sequences aligned to identify the fungus.

Preparation of Cell-Free Extract and Chromatographic Separation. The cell-free extract was prepared by filtering the incubated culture grown in Sabouraud broth (dextrose 4%, peptone 1%) through muslin cloth, resuspending the mycelial pellet in deionized water, and sonicating the mixture in a Branson sonifier. The milky fluid was extracted three times with an equal volume of $CHCl_3$–MeOH (4:1), after which the organic solvent was removed by rotary evaporation at 30 °C, yielding the organic extract. HPLC separation was performed using a Luna RP-18 column (2 mm i.d., length 150 mm, particle size 3μm) and a guard column (Phenomenex, Torrance, CA) at a flow rate of 200 μL min^{-1} at 30 °C. A 10 μL amount of sample was injected in $CHCl_3$–MeOH (4:1). The mobile phases water (A) and acetonitrile (B) were changed in the following manner: 0–5 min 90% A and 10% B, 5–20 min 40% A and 60% B, 20–30 min 2% A and 98% B, 30–32 min 2% A and 98% B, and 35–37 min 90% A and 10% B v/v. The UV signal was recorded at $\lambda = 256$ nm. The retention time of **1** was 20.15 min.

In Vitro Cytotoxicity against Human Cancer Cell Lines. Three selected cancer cell lines (A-549 for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer) were grown for 24 h on 96-well tissue culture plates. Incubation was continued for another 48 h after addition of the test material dissolved in DMSO (final concentration of DMSO <1%) into each well except for the wells that acted as a control or wherein a known drug was added. Cell growth was terminated by addition of trichloroacetic acid. Cells were stained with sulforhodamine B (SRB). Excess dye was removed by washing with water. The bound dye was dissolved in tris-buffer and read using ELISA.

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Supporting Information Available: Conditions of fungal growth and physical and spectroscopic data for camptothecin (**1**) obtained. This information is available free of charge over the Internet at http://pubs.acs.org.

References and Notes

(1) Kauh, E. A.; Bjornsti, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6299–6303.
(2) Potmesil, M.; Pinedo, H. M. *Camptothecins: New Anticancer Agents*; CRC Press: Boca Raton, 1995; pp 123–138.
(3) Swada, S.; Yokokura, T.; Miyasaka, T. *Curr. Pharm. Des.* **1995**, *11*, 113–116.
(4) Torck, M.; Pinkas, M. *J. Pharm. Belg.* **1996**, *51*, 200–203.
(5) Bendixen, C.; Thomsen, B.; Alsner, J.; Westergaard, O. *Biochemistry* **1990**, *29*, 5613–5619.
(6) Meng, L. H.; Liao, Z. Y.; Pommier, Y. *Curr. Top. Med. Chem.* **2003**, *3*, 305–320.
(7) Wall, M. E.; Wani, M. C.; Cooke, C. E.; Palmer, K. T.; McPhail, A. T.; Sim, G. A. *J. Am. Chem. Soc.* **1966**, *88*, 3888–3890.
(8) Cai, C. J.; Hutchinson, C. R. In *The Alkaloids*; Brossi, A., Manske, R. H. F., Eds.; Academic Press: New York, 1983; Vol. 21, Chapter 4, pp 101–137.
(9) Oberlies, N. H.; Kroll, D. J. *J. Nat. Prod.* **2004**, *67*, 129–135.
(10) Cragg, G. M.; Newman, D. J. *J. Nat. Prod.* **2004**, *67*, 232–244.
(11) Becker, Y.; Olshevsky, U. *Isr. J. Med. Sci.* **1973**, *9*, 1578–1581.
(12) Kelly, D. C.; Avery, R. J.; Dimmock, N. J. *J. Gen. Virol.* **1974**, *25*, 427–432.
(13) Pirillo, A.; Verotta, L.; Gariboldi, P.; Torregiani, E.; Bombardelli, E. *J. Chem. Soc., Perkin Trans. 1* **1995**, 583–587.
(14) Govindachari, T. R.; Viswanathan, N. *Indian J. Chem.* **1972**, *10*, 453–454.
(15) Govindachari, T. R.; Ravindranath, K. R.; Viswanathan, N. *J. Chem. Soc., Perkin Trans. 1* **1974**, 1215–1217.
(16) Zhang, X.; Bao, J. Chinese Patent 1,045,266, 1990.
(17) Wall, M. E.; Wani, M. C.; Nicholas, A. W.; Manikumar, G.; Tele, C.; Moore, L.; Truesdale, A.; Leitner, P.; Besterman, J. M. *J. Med. Chem.* **1993**, *36*, 2689–2700.
(18) Rama Rao, A. V.; Yadav, J. S.; Muralikrishna, V. *Tetrahedron Lett.* **1994**, *35*, 3613–3616.
(19) Fang, F. G.; Xie, S.; Lowery, M. W. *J. Org. Chem.* **1994**, *59*, 6142–5143.
(20) Puri, S. C.; Verma, V.; Amina, T.; Qazi, G. N.; Spiteller, M. Indian and PCT application No. 469 NF/02, 2002.
(21) Stierle, A.; Strobel, G.; Stierle, D. *Science* **1993**, *260*, 214–216.
(22) Strobel, G.; Daisy, B.; Castillo, U.; Harper, J. *J. Nat. Prod.* **2004**, *67*, 257–268.
(23) van Hengel, A. J.; Harkes, M. P.; Wichers, H. J.; Hesselink, P. G. M.; Buitelaar, R. M. *Plant Cell, Tissue Organ Cult.* **1992**, *28*, 11–18.
(24) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.
(25) Cappiccino, J. G.; Sherman, N. *Microbiology, a Laboratory Manual*, 6th ed.; The Benjamin/Cummings Publishing Co.: Menlo Park, CA, 1996; pp 129–182.
(26) Vainio, E. J.; Korhonen, K.; Hantula, J. *Mycol. Res.* **1998**, *102*, 187–192.

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