Rapid Communications

An Endophytic Fungus from *Camptotheca acuminata* That Produces Camptothecin and Analogues

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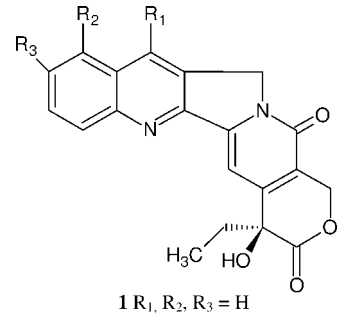
Abstract: The pentacyclic quinoline alkaloid camptothecin (**1**) is a potent antineoplastic agent. Two of its analogues, 9-methoxycamptothecin (**2**) and 10-hydroxycamptothecin (**3**), exhibit similar potency but do not have the potential therapeutic drawbacks produced by unmodified **1**. We have established methodology for the isolation and unequivocal identification and characterization of a novel endophytic fungus isolated from the inner bark of the medicinal plant *Camptotheca acuminata*, which produced **1**−**3** in rich mycological medium (Sabouraud dextrose broth), under shake-flask fermentation conditions. The fungus was identified by its morphology and authenticated by ITS analysis (ITS1 and ITS2 regions and the intervening 5.8S rDNA region). Camptothecin (**1**) and its analogues were identified by ^{1}H NMR spectroscopy and LC-HRMS and confirmed by comparison with authentic standards. The production pattern of the metabolites over seven successive subculture generations of this endophyte was studied. A sharp attenuation in the production of **1** and **2** was observed from the first- through to the seventh-generation subculture. Therefore, these results offer a caution as to the possibility of using endophytic fungi as alternate sources of plant secondary metabolite production. Further studies have been initiated on the analysis of the upstream metabolic intermediates to understand the steps at which the production of the metabolites in question is constrained.

Camptothecin (CPT) (**1**), a pentacyclic quinoline alkaloid, is a potent antineoplastic agent, which was first isolated by Wall and Wani et al.[1] from the wood of *Camptotheca acuminata* Decaisne (Nyssaceae), a plant native to mainland China. The promising results of compound **1** as an antitumor agent in animal models led to its evaluation in the clinic.[2] This potency of compound **1** is by virtue of a unique mechanism of action involving interference with eukaryotic DNA.[3−6] This naturally occurring enantiomer primarily targets the intranuclear enzyme DNA topoisomerase I (Topo I), which is required for the swivelling and relaxation of DNA during molecular events, namely, DNA replication and transcription.[7] CPT (**1**) also hinders the synthesis of RNA.[8] A number of reports have been published indicating the therapeutic potential of compound **1**,[9] against colon cancer,[10] AIDS,[11] uterine, cervical, and ovarian cancer,[12] and malaria.[13]

The promising potency and efficacy of unmodified **1** is, however, compromised in therapeutic applications due to its very low solubility in aqueous media and high toxicity.[9,14] Compound **1** undergoes rapid inactivation through lactone ring cleavage at physiological pH to form the water-soluble carboxylate, which is inactive and readily binds to human serum albumin (HSA), making it inaccessible for cellular uptake.[15,16] Moreover, the sodium salt of **1** (more water soluble) is filtered by the kidneys and causes hemorrhagic cystitis and myelotoxicity, rendering it unsuitable for clinical trials.[2] Additionally, the half-life of unmodified CPT (**1**)-induced Topo I-mediated DNA breakage is far less than those of modified camptothecin derivatives.[17] Although compound **1** suffers from these drawbacks, its typical action-mechanism and specific target have stimulated intensive efforts to identify and develop various analogues (mainly by synthetic and semisynthetic routes) to overcome the drawbacks of unmodified CPT (**1**), yet retain its potency. Extensive studies on the structure−activity relationships (SAR) of compound **1** (Figure S1, Supporting Information) have led to the formulation of various important analogues having different potential benefits over CPT (**1**).[9,14]



1 R_1, R_2, R_3 = H
2 R_1, R_3 = H; R_2 = OCH_3
3 R_1, R_2 = H; R_3 = OH

9-Methoxycamptothecin (**2**) and 10-hydroxycamptothecin (**3**) are two important analogues of compound **1** that hold potential for their anticancer efficacy[18,19] and have been reported to inhibit Topo I.[20] Compounds **2** and **3** belong to the class of C-9/C-10 (R_2/R_3)-substituted CPT analogues, some of which have already entered clinical trials against various malignant diseases.[14,21,22] Substitution at the C-9 or C-10 position with suitable groups induces superior antitumor activity.[23] The SAR studies show a close correlation between an ability to inhibit Topo I and overall cytotoxic potency based on the substitution at a particular position (Figure S1, Supporting Information). In general, substitutions at C-7, C-9, and C-10 (on the quinoline ring, i.e., ring A or B) tend to increase Topo I inhibition in addition to conferring increased water solubility. Moreover, the differences in the percentage present in the lactone form at equilibrium is related to steric considerations of the various substituents at the C-9 (R_2) and C-10 (R_3) positions.[14] This is because substituents at these positions cause steric hindrance and prevent binding of the carboxylate forms to HSA, and so drive the equilibrium toward the lactone species. Compounds **2** and **3** have a methoxy and a hydroxy group at the C-9 and C-10 (R_2 and R_3) position, respectively, and these account for their potential therapeutic advantage over compound **1**.[14,22]

Compounds **2** and **3** are not abundant and are accumulated only in relatively low concentrations in various plant species that are screened for CPT (**1**), mainly *Camptotheca acuminata* and *Nothapodytes nimmoniana* (J. Graham) Mabb. (Icacinaceae), and are available only in combination with many other camptothecin derivatives. Hence, their isolation would require tremendous effort to separate the mixtures of many camptothecin derivatives and in minor quantities, with few alternatives. Therefore, it is desirable to develop strategies for sustainable production of **1**−**3** from an alternate source (involving a microorganism) by means of fermentation technology.

Herein we report, for the first time, the production of camptothecin (**1**), 9-methoxycamptothecin (**2**), and 10-hydroxycamptothecin

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(**3**) by an endophytic fungus, INFU/Ca/KF/3, isolated from *Camptotheca acuminata*, a plant specimen obtained from the campus of the Southwest Forestry University (SWFU), Kunming (Yunnan Province), People's Republic of China (Figure S2, Supporting Information). The fungus has been identified as *Fusarium solani* (Figure S3, Supporting Information) based on its morphology and authenticated by the molecular analysis of the ITS region of rDNA containing ITS1, and ITS2, and the intervening 5.8S rRNA gene. Since *F. solani* from other sources do not produce **1**, **2**, or **3**, it can be presumed that the inclusion of gene(s) responsible for the production of these metabolites into the fungal genome has been obtained from the host by means of horizontal gene transfer. Therefore, differences in the genetic makeup of endophytic INFU/Ca/KF/3 and *F. solani* isolated from other sources are evident. The ITS-5.8S rDNA sequence (S4, Supporting Information) obtained has been deposited into EMBL-Bank (European Molecular Biology Laboratory) under accession number FM179605. In turn, the endophytic fungus has been deposited at the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, DSMZ), Braunschweig, Germany (accession number DSM 21921). The potential of using endophytes as an effective alternative or novel source for therapeutic compounds has been recognized. Several workers have reported the use of endophytes for the production of pacitaxel, camptothecin, and podophyllotoxin isolated from the hosts *Taxus brevifolia*,[24] *Nothapodytes foetida*,[25,26] and *Podophyllum peltatum* L. (Berberidaceae),[27] respectively, although there are no reports of successful industrial scale-ups. There is no published report that **2** or **3** might be produced by any microorganism associated with *Camptotheca acuminata* or any other plant species.

Both the fungal biomass and the culture media from grown cultures were assessed for the presence of **1**−**3**. The culture media did not yield any trace of these compounds. The identification of the compounds in the fungal biomass was achieved using LC-HRMS, LC-HRMS,^{2} and LC-HRMS^3 and by comparison with the authentic reference standards. The characteristic fragments of MS^3, together with a brief interpretation of the fragments and the comparison of the retention times (TICs), are presented in Figure 1. The reference standards used were **1** from Sigma-Aldrich Chemie GmbH, Steinheim, Germany, and **3** from LKT Laboratories Inc., St. Paul, MN. Unfortunately, the 9-methoxycamptothecin (**2**) produced by the cultured endophyte was not successfully elucidated by LC-NMR due to interference from other metabolites in the fungal extract that were inseparable. Hence, **2** was first isolated and established as an authentic standard from *N. nimmoniana* plant (which contains considerable amounts of **2**)[28,29] of Indian origin (Western Ghats, India) using LC-HRMS^3 and ^{1}H NMR spectroscopy in order to elucidate the exact position of the methoxy group (S5, Supporting Information), and thereafter fungal **2** was confirmed. Furthermore, we synthesized 10-methoxycamptothecin from standard **3** by its reaction with an ether solution of diazomethane (30 min), which was identical to the 10-methoxycamptothecin from the host *C. acuminata* plant (S5, Supporting Information). It can be seen from Figure 1(m and n) that there is a difference not only in the retention times between 9-methoxycamptothecin (**2**) and 10-methoxycamptothecin but also in the intensities of the MS^3 fragments (*m*/*z* 320). Interestingly, an additional isomeric-hydroxycamptothecin was accumulated by the cultured endophyte in addition to **3** in some generations only, having a different retention time (t_R) than **3** (t_{R3} 22.29 min, $t_{\text{Risomeric-hydroxycamptothecin}}$ 22.95 min) but identical mass spectra, as shown in Figure 1(h). It is most probably the 9-hydroxycamptothecin isomer, which will be confirmed in the future.

A detailed study of metabolite production was undertaken over generations (Figure 2). In shake-flask incubations of the endophytic fungus, we found an inverse relation between the hyphal biomass and the respective CPT (**1**) and 9-methoxycamptothecin (**2**) production across the first to the seventh generation. The inverse relation was strong from the third to the seventh generation, where the levels of **1** and **2** were substantially attenuated. Interestingly, the endophytic production of 10-hydroxycamptothecin (**3**) was detected only from the fourth generation, which remained almost constant through to the seventh generation. However, compound **3** could not be quantified because the production up to the third generation was below the limit of detection (<LOD) and from the fourth to seventh generation was below the limit of quantitation (<LOQ). With respect to CPT and its analogues, the current study is also the first of its kind to report an attenuation of CPT production upon subculturing an endophytic fungus.

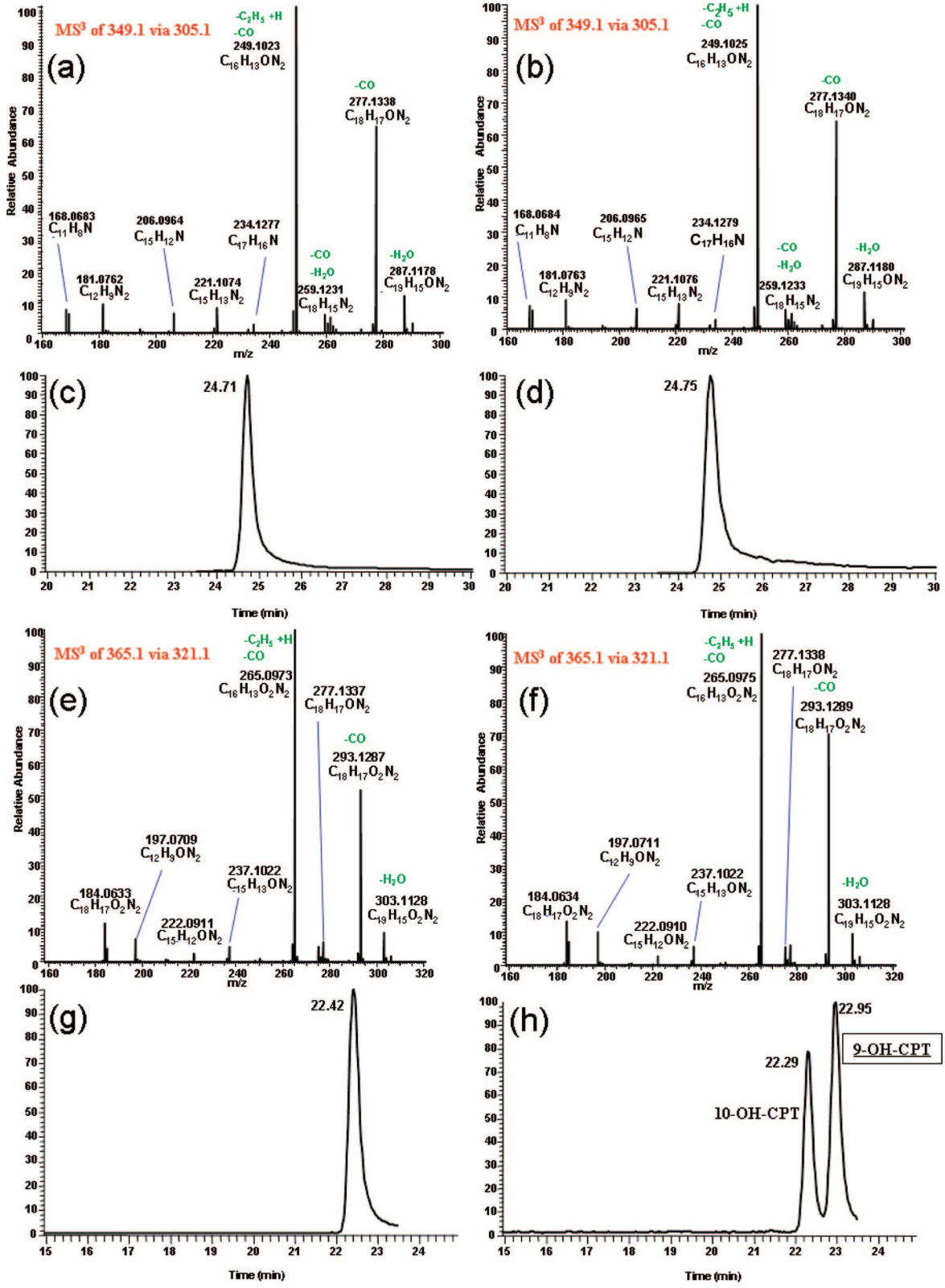
Attenuation or dampening of metabolite production through subculture generation has been one of the vexing issues in exploiting endophytic fungi as sources of novel metabolites. It may be conjectured that the lack of host stimuli in the axenic cultures could be one of the reasons setting off the attenuation. However, the exact role of the host stimuli (host extract) in the reversal of the attenuation has not been elucidated. Our results seem to suggest a tradeoff over energy (nutrient) allocation between growth (gain in biomass) on one hand and production (gain in metabolite) on the other. Thus, the isolate INFU/Ca/KF/3, which is otherwise genetically provisioned to produce high levels of **1** and two of its important analogues, tends to have lesser nutrient available for growth and vice versa. The explanation for the observed production of **3** only during the later generations at the cost of attenuation of **1** and **2** could lie in the understanding of the biosynthetic pathway of CPT. It is highly probable that there might be a split in the biochemical pathway somewhere along the lines of CPT (**1**) production, wherein a precursor destined to form CPT (**1**) receives a hydroxy group at some intermediate step to form **3**. Alternatively, **3** could be a post-metabolic product of **1** (produced by the endophyte). While evidence to support these hypotheses is currently not available, detailed biochemical mass-balance studies of the pathway(s) in question in the endophyte will shed light on this enigmatic observation. We have already initiated studies on the analysis of the upstream metabolic intermediates to understand the steps at which the production of the metabolites in question is constrained.

Experimental Section

General Experimental Procedures. Compounds **1**−**3** were identified and quantified by LC-HRMS and LC-HRMS^3 fragment spectra (LTQ-Orbitrap spectrometer, Thermo Scientific), which were consistent with the reference standards. The mass spectrometer was equipped with a Dionex HPLC system Ultimate 3000 consisting of pump, flow manager, and autosampler (injection volume 0.6 μL). Nitrogen was used as sheath gas (6 arbitrary units), and helium served as the collision gas. The separations were performed by using a Phenomenex Gemini C_{18} column (3 μm, 0.3 × 150 mm) (Torrance, CA) with a H_2O (+ 0.1% HCOOH) (A)/acetonitrile (+ 0.1% HCOOH) (B) gradient (flow rate 4 μL min^{-1}). Samples were analyzed by using a gradient program as follows: 95% A isocratic for 5 min, linear gradient to 60% A within 12 min, and to 100% B in 29 min. After 100% B isocratic for 5 min, the system returned to its initial condition (95% A) within 1 min and was equilibrated for 7 min. The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 200−800) with nominal mass resolving power of 60 000 at *m*/*z* 400 with a scan rate of 1 Hz, with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using one internal lock mass; *m*/*z* 391.284286; bis-(2-ethylhexyl)-phthalate. MS^2 leads to the corresponding CO_2 loss of the precursor (CID of 45). The final MS^3 measurement was performed under CID of 45 and resulted in characteristic fragments of the compounds (Figure 1). The ^{1}H NMR measurements for **2** and 10-methoxycamptothecin were made at 298 K with a Bruker DRX-400 spectrometer using 5 mm tubes with $CDCl_3$ (Merck, Darmstadt, Germany) as solvent (S5, Supporting Information).

Isolation and Culture of Endophytic Fungi. As part of an effort to identify endophytic fungi that produce **1** and its analogues, inner bark explanted from a fully matured *C. acuminata* tree was collected

(August 2007) from the Southwest Forestry University (SWFU) campus, Kunming, Yunnan Province, People's Republic of China. This specimen is presently being maintained at the Southwest Forestry University [Figure S2(a and b), Supporting Information]. Each of the explants (inner bark) was carefully excised from the trunk of the host (Figure S2(c and d), Supporting Information) and collected in clean, dry, plastic bags. The explants were transported to the Institut für Umweltforschung (Institute of Environmental Research, INFU), Technische Universität Dortmund, Dortmund, Germany, immediately, and processed within 48 h of collection. The explants were washed thoroughly in running tap water followed by deionized (DI) water to remove any dirt sticking to them and stored at 4 °C until the isolation procedure. Surface sterilization of the explants was done following our previously established method.[30] Briefly, the explants were thoroughly washed in running tap water, and small fragments of inner bark of the stems of approximately 10 mm (length) by 5 mm (breadth) were cut with the aid of a flame-sterilized razor blade. Then, the small stem fragments were surface-sterilized by sequential immersion in 70% ethanol for 1



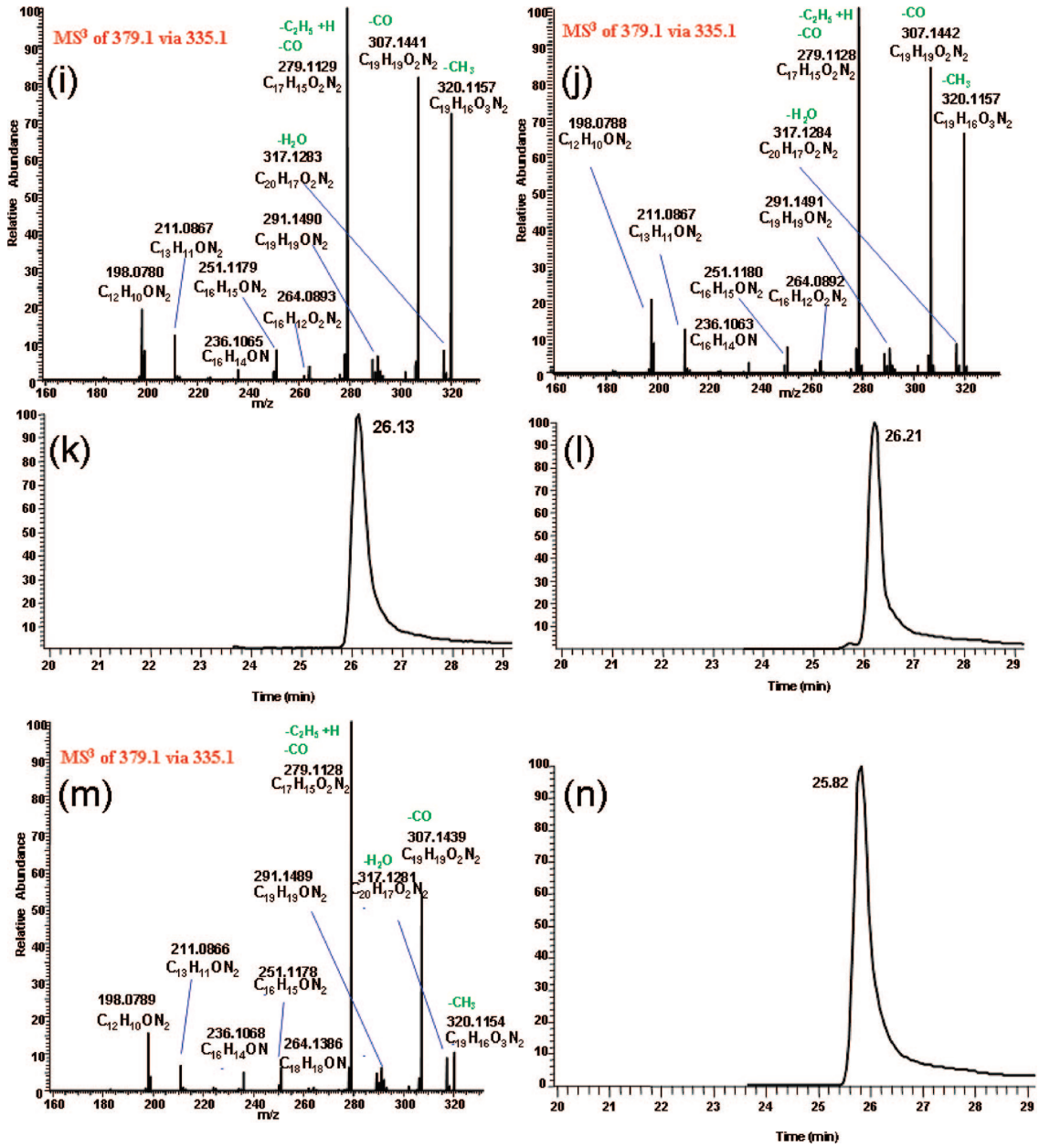


Figure 1. High-resolution MS^3 product ions and TICs indicating the retention times, respectively, of (a and c) standard camptothecin (**1**); (b and d) fungal camptothecin (**1**); (e and g) standard 10-hydroxycamptothecin (**3**); (f and h) fungal 10-hydroxycamptothecin (**3**); (i and k) standard 9-methoxycamptothecin (**2**); (j and l) fungal 9-methoxycamptothecin (**2**); (m and n) 10-methoxycamptothecin (from *Camptotheca acuminata* host plant). Detailed explanations are provided in the text.

min, 1.3 M sodium hypochlorite (3−5% available chlorine) for 3 min, and 70% ethanol for 30 s. Finally, these surface-sterilized stem pieces were rinsed three times in sterile, double-distilled water for 1 min each, to remove excess surface sterilants. The excess moisture was blotted on a sterile filter paper. Surface-sterilized stem fragments, thus obtained, were evenly spaced in Petri dishes (TPP, Trasadingen, Switzerland) containing water agar (WA) medium (DIFCO, cat. no. 214530) amended with streptomycin (100 mg L^{-1}) to eliminate any bacterial growth. Petri dishes were sealed using Parafilm (Pechiney, Chicago, IL) and incubated at 28 ± 2 °C in an incubator until fungal growth started. To ensure proper surface sterilization, unsterilized stem segments were prepared simultaneously and incubated under the same conditions in parallel to isolate the surface-contaminating fungi. The cultures were monitored every day to check the growth of endophytic fungal colonies from the sample segments. The hyphal tips, which grew out from sample segments over 4−6 weeks, were isolated and subcultured onto a rich mycological medium, Sabouraud dextrose agar (SA) medium (DIFCO, cat. no. 210950), and brought into pure culture. Altogether, 11 putative endophytes were isolated, of which only one was able to produce **1**, **2**, and **3** and was taken up for further studies. The axenic culture, thus obtained, was coded as INFU/Ca/KF/3 and preserved by lyophilization, as well as by cryopreservation at −70 °C in the microbial library of our institute.

Identification of the Endophytic Isolate. The endophytic fungus was grown on SA for 5 days at 28 ± 2 °C and morphologically characterized (S6, Supporting Information). The mycelium was scraped directly from the surface of the agar culture (5 days old) and weighed. Nucleic acid was extracted and purified using the AppliChem DNA isolation kit for genomic DNA (AppliChem GmbH, Darmstadt, Germany) using the Chomczynski method,[31] suitably modified. For identification and differentiation, the Internal Transcript Spacer regions (ITS1 and ITS2) and the intervening 5.8S rRNA region was amplified and sequenced using electrophoretic sequencing on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) using BigDye Terminator v 3.1 cycle sequencing kit. The ITS regions of the fungus were amplified using PCR (PeqStar thermocycler, PeqLab GmbH, Erlangen, Germany) and the universal ITS primers, ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT

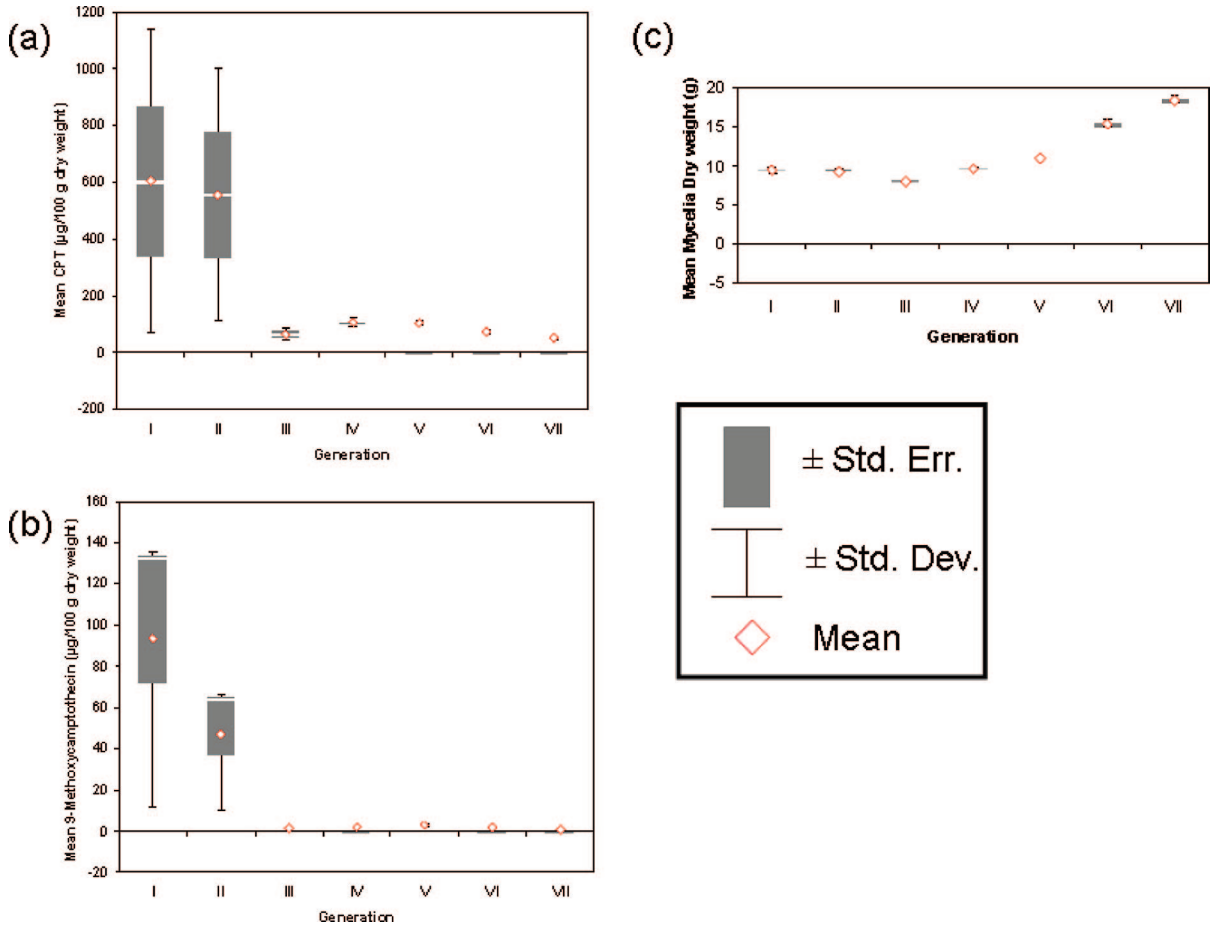


Figure 2. Box and Whisker's plot of the metabolite production pattern by the endophytic fungal isolate from the 1st to the 7th subculture generation under shake-flask conditions and their correlation with the fungal biomass accumulation. (a) Mean CPT (**1**). (b) Mean 9-methoxycamptothecin (**2**). (c) Mean fungal biomass dry weight. All values represent the mean of independent experiments in triplicate. The fungal 10-hydroxycamptothecin (**3**) was <LOD up to the 3rd generation and <LOQ from the 4th to the 7th generation.

GC-3′). The PCR products were purified and desalted using the Chargeswitch purification kit (Invitrogen, Carlsbad, CA) and sequenced on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). The sequences were aligned and prepared with the software DNAstar Lasergene SeqMan (Madison, WI) and matched against the nucleotide-nucleotide database (BLASTn) of the U.S. National Center for Biotechnology Information (NCBI) for final identification of the endophytic isolate.

Preparation of Cell-Free Extract. Shake-flask fermentations were performed with the fungus under specific conditions, and the special morphological features under the submerged culture conditions were noted (S7, Supporting Information). The cell-free extract was prepared by filtering the incubated culture through muslin cloth under vacuum. The mycelia and broth were treated separately. The mycelial pellet was dried in an oven (25 °C) to obtain the dry weight and was resuspended in deionized water (DI). This suspension was then sonicated in an ultrasonicator (Branson B-12, Danbury, CT) under chilled conditions. The milky fluid, thus obtained, was extracted three times with 50 mL of $CHCl_3$−MeOH (4:1). The organic solvent was removed after each extraction by rotary evaporation under vacuum at 30 °C, yielding the organic extract. The spent broth (100 mL) was extracted directly in the same way. To ensure the production of compound **2** by the cultured endophyte, extractions were performed with $CHCl_3$−CD_3OD (4:1) in a similar fashion in parallel and analyzed. There was no incorporation of the CD_3OD into the CPT moiety.

Generation Studies on the Endophytic Isolate. In order to establish the production pattern of **1**, **2**, and **3** over successive generations, a study was devised to understand the variance of metabolite production from one generation to another and to correlate that with the fungal growth pattern. Briefly, the established axenic isolate INFU/Ca/KF/3 was subcultured from the first generation using the hyphal-tip method to obtain the second-generation isolate. Subsequent subcultures were made in a similar way to obtain up to the seventh generation of the endophytic isolate. Shake-flask fermentations were performed with the isolates for each generation, and the extraction and metabolite analysis were performed using the methods detailed above. The results were statistically analyzed using the Box and Whisker's plot method.

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Supporting Information Available: Structure−activity relationship (SAR) of CPT (**1**), structural elucidation and establishment of 9-methoxycamptothecin (**2**) as standard from *Nothapodytes nimmoniana* plant using ^{1}H NMR spectroscopy, *Camptotheca acuminata* plant maintained at the Southwest Forestry University (SWFU) campus, Kunming, People's Republic of China, and its endophytic fungus, INFU/Ca/KF/3, growing on rich mycological medium; morphological descriptions of the isolated endophytic fungus on solid rich media (SA), in culture (SB) including culture conditions; the ITS-5.8S rDNA sequence obtained from the endophytic fungus INFU/Ca/KF/3 and a dendrogram showing the phylogenetic position of the isolate. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmer, K. H.; McPhail, A. T.; Sim, G. A. *J. Am. Chem. Soc.* **1966**, *88*, 3888–3890.
(2) Moertel, C. G.; Schutt, A. J.; Reitemeier, R. J.; Hahn, R. G. *Cancer Chemother. Rep.* **1972**, *56*, 95–95.
(3) Kauh, E. A.; Bjornsti, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6299–6303.
(4) Potmesil, M.; Pinedo, H. M. *Camptothecins: New Anticancer Agents*; CRC Press: Boca Raton, FL, 1995.
(5) Sawada, S.; Yokokura, T.; Miyasaka, T. *Curr. Pharm. Des.* **1995**, *1*, 113–132.
(6) Torck, M.; Pinkas, M. *J. Pharm. Belg.* **1996**, *51*, 200–207.
(7) Ling-Hua, M.; Zhi-Yong, L.; Pommier, Y. *Curr. Top. Med. Chem.* **2003**, *3*, 305–320.
(8) Bendixen, C.; Thomsen, B.; Alsner, J.; Westergaard, O. *Biochemistry* **1990**, *29*, 5613–5619.
(9) Li, Q. Y.; Zu, Y. G.; Shi, R. Z.; Yao, L. P. *Curr. Med. Chem.* **2006**, *13*, 1–19.
(10) Giovanella, B. C.; Stehlin, J. S.; Wall, M. E.; Wani, M. C.; Nicholas, A. W.; Liu, L. F.; Silber, R.; Potmesil, M. *Science* **1989**, *246*, 1046–1048.
(11) Priel, E.; Showalter, S. D.; Blair, D. G. *AIDS Res. Hum. Retrovir.* **1991**, *7*, 65–72.
(12) Takeuchi, S.; Dobashi, K.; Fujimoto, S.; Tanaka, K.; Suzuki, M.; Terashima, Y.; Hasumi, K.; Akiya, K.; Negishi, Y.; Tayama, T. *Gan To Kagaku Ryoho* **1991**, *18*, 1681–1689.
(13) Bodley, A. L.; Cumming, J. N.; Shapiro, T. A. *Biochem. Pharmacol.* **1998**, *55*, 709–711.
(14) Kehrer, D. F. S.; Soepenberg, O.; Loos, W. J.; Verweij, J.; Sparreboom, A. *Anti-Cancer Drugs* **2001**, *12*, 89–105.
(15) Fassberg, J.; Stella, V. J. *J. Pharm. Sci.* **1992**, *81*, 676–684.
(16) Burke, T. G.; Mi, Z. *Anal. Biochem.* **1993**, *212*, 285–287.
(17) Holden, J. A.; Wall, M. E.; Wani, M. C.; Manikumar, G. *Arch. Biochem. Biophys.* **1999**, *370*, 66–76.
(18) Wu, T. S.; Leu, Y. L.; Hsu, H. C.; Ou, L. F.; Chen, C. C.; Chen, C. F.; Ou, J. C.; Wu, Y. C. *Phytochemistry* **1995**, *39*, 383–385.
(19) Sawada, S.; Yokokura, T.; Miyasaka, T. *Ann. N.Y. Acad. Sci.* **1996**, *803*, 13–28.
(20) Zhou, B. N.; Hoch, J. M.; Johnson, R. K.; Mattern, M. R.; Eng, W. K.; Ma, J.; Hecht, S. M.; Newman, D. J.; Kingston, D. G. I. *J. Nat. Prod.* **2000**, *63*, 1273–1276.
(21) Stehlin, J. S.; Giovanella, B. C.; Natelson, E. A.; De Ipolyi, P. D.; Coil, D.; Davis, B.; Wolk, D.; Wallace, P.; Trojacek, A. *Int. J. Oncol.* **1999**, *14*, 821–831.
(22) Tanizawa, A.; Fujimori, A.; Fujimori, Y.; Pommier, Y. *J. Natl. Cancer Inst.* **1994**, *86*, 836–842.
(23) Huang, M.; Gao, H.; Chen, Y.; Zhu, H.; Cai, Y.; Zhang, X.; Miao, Z.; Jiang, H.; Zhang, J.; Shen, H.; Lin, L.; Lu, W.; Ding, J. *Clin. Cancer. Res.* **2007**, *13*, 1298–1307.
(24) Stierle, A.; Strobel, G. A.; Stierle, D. *Science* **1993**, *260*, 214–216.
(25) Puri, S. C.; Verma, V.; Amna, T.; Qazi, G. N.; Spiteller, M. *J. Nat. Prod.* **2005**, *68*, 1717–1719.
(26) Rehman, S.; Shawl, A. S.; Kour, A.; Andrabi, R.; Sudan, P.; Sultan, P.; Verma, V.; Qazi, G. N. *Appl. Biochem. Microbiol.* **2008**, *44*, 203–209.
(27) Eyberger, A. L.; Dondapati, R.; Porter, J. R. *J. Nat. Prod.* **2006**, *69*, 1121–1124.
(28) Ramesha, B. T.; Amna, T.; Ravikanth, G.; Gunaga, R. P.; Vasudeva, R.; Ganeshaiah, K. N.; Shaanker, R. U.; Khajuria, R. K.; Puri, S. C.; Qazi, G. N. *J. Chromatogr. Sci.* **2008**, *46*, 362–368.
(29) Namdeo, A. G.; Sharma, A.; Mahadik, K. R. *Pharmacogn. Rev.* **2008**, *2*, 110–115.
(30) Kusari, S.; Lamshöft, M.; Zühlke, S.; Spiteller, M. *J. Nat. Prod.* **2008**, *71*, 159–162.
(31) Chomczynski, P.; Sacchi, N. *Anal. Biochem.* **1987**, *162*, 156–159.

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