Light-Independent Metabolomics of Endophytic *Thielavia subthermophila* Provides Insight into Microbial Hypericin Biosynthesis

Souvik Kusari,[†] Sebastian Zühlke,[†] Ján Košuth,[‡] Eva Čellárová,[‡] and Michael Spiteller*,[†]

Institut für Umweltforschung (INFU), Technische Universität Dortmund, Otto-Hahn-Strasse 6, 44221 Dortmund, Germany, and Institute of Biology and Ecology, Faculty of Science, P. J. Šafárik University in Košice, Mánesova 23, 041 54 Košice, Slovakia

The possible microbial mechanism of hypericin (**1**) and emodin (**2**) biosynthesis was studied in axenic submerged culture conditions in the endophytic fungus *Thielavia subthermophila*, isolated from *Hypericum perforatum*. The growth and secondary metabolite production of the endophyte remained independent of the illumination conditions. This production remained unaltered on spiking the medium with 3 or 5 mM **2**, although the biomass accumulation was reduced. Neither emodin anthrone (**3**) nor protohypericin (**4**) could be detected at any stage of fermentation, irrespective of either spiking or illumination conditions. The endophytic metabolites exhibited photodynamic cytotoxicity against the human acute monocytic leukemia cell line (THP-1), at 92.7 vs 4.9%, and 91.1 vs 1.0% viability by resazurin and ATPlite assays, in light and in the dark, respectively. In trying to ascertain the presence/expression of the candidate *hyp-1* gene in the endophyte, it was revealed that the *hyp-1* gene was absent in *T. subthermophila*, indicating that the biosynthetic pathway in the endophytic fungus might be different and/or governed by a different molecular mechanism than the host plant or host cell suspension cultures. We have discussed the biosynthetic principles and evolutionary implications relating to endophytic *T. subthermophila* based on the results obtained.

Plant species of the genus *Hypericum* have long been used as traditional medicinal plants in various parts of the world on account of their therapeutic efficacy.[1] Their main constituents are napthodianthrones, primarily represented by hypericin (**1**) and pseudohypericin; flavonoids such as hyperoside, rutin, quercetin, and quercitrin; and a third group of phloroglucinol derivatives such as hyperforin and adhyperforin.[2] Among the myriad of *Hypericum* species, *H. perforatum* L. (Clusiaceae), commonly called St. John's wort, has been one of the most important and commercially recognized species. *H. perforatum* is a pseudogamous, facultatively apomictic, perennial medicinal plant native to Europe, West and South Asia, North Africa, North America, and Australia,[3,4] which has long been in use in traditional, ayurvedic, and folk medicine. This legacy of St. John's wort has been attributed mainly to the naphthodianthrone derivative hypericin, which is exploited in modern phytotherapeutic applications as an antidepressant,[2,5] in wound-healing, for its anti-inflammatory,[6] antimicrobial, and antioxidant properties,[7] for the relief of sinusitis[8] and seasonal affective disorders,[9] and for its antiviral potency.[10] Several in vitro studies have revealed the multifaceted cytotoxic activity of hypericin (**1**) on cancer cells as a result of its photodynamic activity.[10−13]

A hypothetical biosynthetic pathway was proposed shortly after the isolation and characterization of hypericin (**1**) extracted from *H. perforatum*.[14−17] By isolating some intermediate compounds, these workers formulated a theoretical polyketide pathway based largely on the paradigm that chemical principles should apply to biosynthetic processes (S1, Supporting Information). A schematic representation of the proposed polyketide pathway is depicted in Figure 1a. In the case of hypericin (**1**) biosynthesis, the homodimeric type III PKS had earlier been suggested to be responsible for the condensation of one molecule of acetyl CoA with seven molecules of malonyl CoA to form an octaketide chain that subsequently undergoes cyclizations and decarboxylation, leading to the formation of emodin anthrone (**3**),[18,19] the first cyclization product of the polyketide pathway. However, the type III PKS with octaketide synthase (OKS) activity responsible for the formation of **3** has not

been characterized as yet, although a PKS named HpPKS2 has recently been reported to catalyze the condensation of one acetyl CoA with two to seven malonyl CoA to yield tri- to octaketide products, but not **3**.[20] It is postulated that a dianthrone would arise from **3**, probably by oxidative coupling of the anthranol, and would lead by further oxidation of its enol form to a dehydrodianthrone and then, from a helianthrone derivative [protohypericin (**4**)], to yield **1**.[17] Compound **4** is readily converted to compound **1** upon irradiation with visible light. The knowledge that anthrone−dianthrone interconversion takes place readily, coupled with a basic understanding of how anthracene nuclei are cross-linked by oxidation, contributed to the proposal of the original scheme for the biosynthesis of **1** from **3**.[15−17] On the other hand, Chen et al.[21] characterized the enzyme emodinanthrone-oxygenase that catalyzes the fixation of molecular oxygen into **3** to yield the anthraquinone emodin (**2**). Furthermore, Bais et al.[22] reported the biochemical and molecular characterization of an enzyme, Hyp-1, from dark-grown *H. perforatum* cell cultures, that specifically catalyzes the direct conversion of **2** to **1** in vitro (Figure 1c). More recently, we published a study on the expression of the *hyp-1* gene in different organs of *H. perforatum* seedlings in early stages of development, purporting to locate the sites of biosynthesis of **1**.[23] Our study, however, shows that the sites of biogenesis and accumulation of **1** in the *Hypericum* plants are independent of the expression of the *hyp-1* gene. Concomitantly, the chemical synthesis of **1** follows the pattern of the proposed biogenesis. Compound **3** is the precursor of compound **1** synthesis and is obtained either by the reduction of **2** isolated from the bark of the breaking buckthorn (*Cortex frangulae*)[24] or by synthesizing **2** as first described by Brockmann et al.[25] The synthetic routes to compound **1**[18,24,26] are shown in Figure 1b. Additionally, a new high-yield synthetic route to **3** with commercially available *o*-cresotinic acid as precursor has been developed by Falk and Schoppel.[27]

Recently we isolated an endophytic fungus from the stems of *H. perforatum*, collected from natural populations at Harwan, Jammu and Kashmir, India, that is capable of indigenously producing hypericin (**1**) and emodin (**2**) in submerged axenic culture in vitro.[28] Given the present knowledge about the biosynthetic (in plants) and synthetic routes to **1** and **2**, and the current understanding of the costs and benefits of endophytes in general to the host plants,

* To whom correspondence should be addressed. Tel: +49(0)231-755-4080. Fax: +49(0)231-755-4085. E-mail: m.spiteller@infu.uni-dortmund.de.
† INFU, Technische Universität Dortmund.
‡ P. J. Šafárik University in Košice.
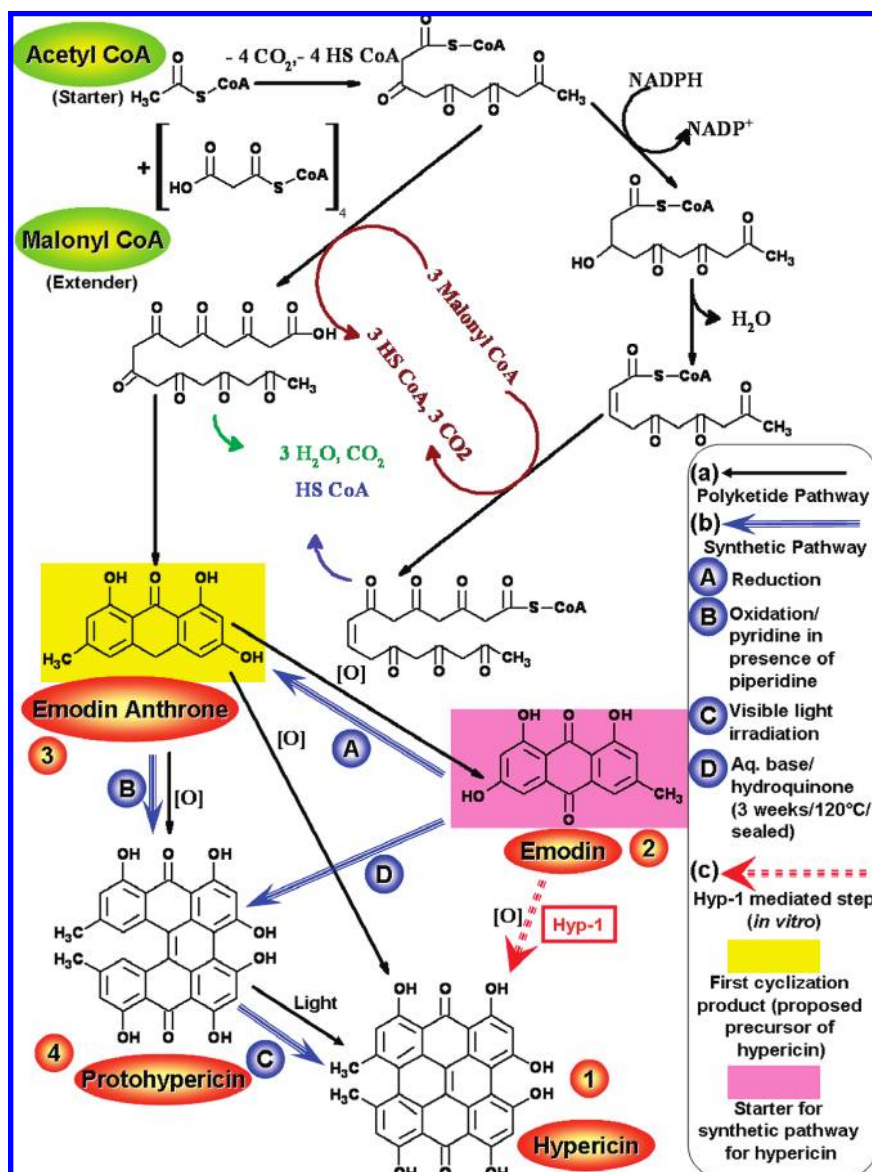
Figure 1. Schematic representation of the different ways of hypericin (**1**) production. (a) Hypothetical polyketide pathway adapted from the originally proposed pathway by Brockmann et al.[16] and Thomson.[17] (b) Synthetic routes of preparing hypericin (**1**). (c) Hyp-1-mediated pathway from emodin (**2**) to hypericin (**1**) as proposed by Bais et al.[22]

the discovery of an endophyte equipped with the necessary molecular machinery to accumulate both **1** and **2** outside its host raises some intriguing questions about the microbial metabolomics. We have performed detailed studies on the growth and production kinetics of the endophyte in the presence and absence of light, the effect of spiking with **2** (the probable precursor of **1** in the hypothetical polyketide pathway, see Figure 1) on the production of **1** and its own accumulation in light and under light protection, and the effect of growth on production and vice versa. Moreover, the cytotoxic and photodynamic effects of fungal **1** and **2** on human acute monocytic leukemia cells (THP-1) were studied. Furthermore, we attempted to ascertain whether the candidate *hyp-1* gene suggested to encode for the coupling protein as the key enzyme in the biosynthesis of **1** in *H. perforatum* cell cultures[22] is present/ expressed in the endophyte as well. We have attempted to elucidate the biosynthetic principles that might be involved in the microbial production of **1** and the evolutionary implications on the basis of the results obtained.

Results and Discussion

Identification of the Endophytic Fungus. The endophytic fungus INFU/Hp/KF/34B, isolated from the stems of *H. perforatum*,

was investigated on the basis of morphological characteristics and by fungus ribotyping [Figure S2, S3(a and b), Supporting Information]. The detailed macroscopic characteristics of the fungus have been reported earlier.[28] Interestingly, the fungus has shown very good rate of growth and survival even up to $38-40$ °C, revealing its thermotolerant capabilities. Microscopic studies of the fungus have revealed that the conidia (aleuriospores, chlamydospores) forming laterally or terminally on the hyphae or on short branches are broadly clavate or pyriform, with a truncate base, single-celled, hyaline or light brown and measure $5-7 \times 3-5$ μm. The ascomata developing within the mycelial mat are spherical, black, $90-200$ μm in diameter. The thin, dark wall of the ascomata is composed of textura epidermoidea or of flattened, in outline irregular, $6-8$ μm sized cells and is often covered with dark hyphae. The ascospores are fusiform or ellipsoidal, single-celled, brown, $14-20$ $\times 8-10$ μm, with a distinct, subapical germ pore. On the basis of these typical features, the fungus could be assigned to the genus *Thielavia*. The final assignment of the species could, therefore, be done by resorting to the molecular analysis of the ITS region of the rDNA containing ITS1, and ITS2, and the intervening 5.8S rRNA gene. The ITS-5.8S rDNA sequence (S3a, Supporting Information) obtained has been deposited into EMBL-Bank (Eu-
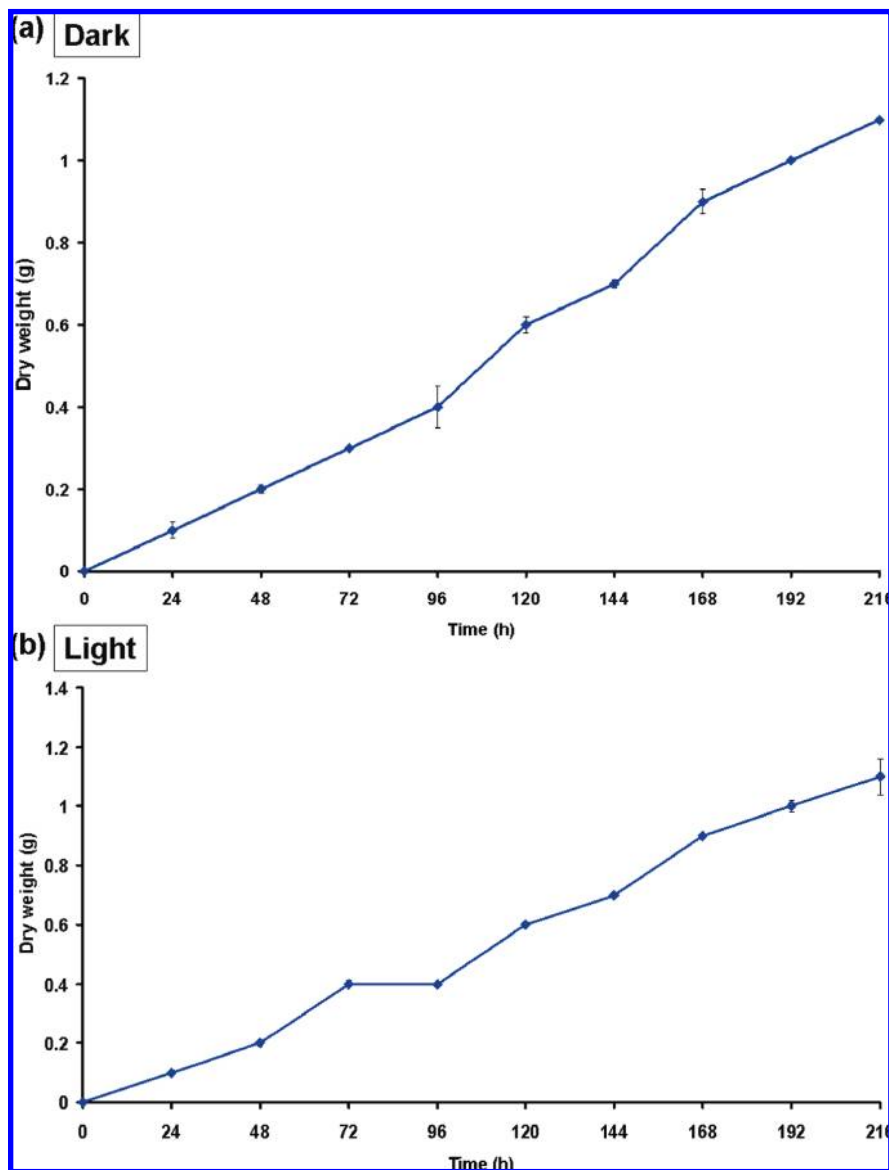
Figure 2. Growth kinetics of the cultured endophyte producing hypericin (**1**) and emodin (**2**). (a) Under light protection. (b) Without light protection.

ropean Molecular Biology Laboratory), accession number AM909688. On the basis of the ITS-5.8S rDNA analysis, in addition to its morphology, the endophytic fungus, INFU/Hp/KF/34B, has been identified as *Thielavia subthermophila* (S3b, Supporting Information).

Growth Kinetics of the Endophyte in the Light and in Darkness. The growth kinetics of the endophytic fungus INFU/Hp/KF/34B was examined up to the ninth day (216 h) of incubation. The endophyte exhibited an exponential increase in the dry weight of mycelia up to the ninth day (216 h) of incubation both in the light and in darkness (Figure 2). The kinetics of growth was similar in both light and dark conditions. Interestingly, the amounts of biomass produced by the fungus after submerged fermentation under light and dark conditions were comparable for each time period throughout the whole experiment. Growth commenced immediately after the fermentation was started. The biomass accumulation at the end of fermentation (216 h) was the same under both conditions. However, even though the fungus exhibited exponential growth when illuminated and in the dark, the relative growth rates at different time points were different in the two situations.

Production Kinetics of the Endophyte in the Light and in Darkness. In order to study the production kinetics of **1** and **2**,

the mycelia were collected every 24 h and metabolites were isolated from both the mycelia and spent broth. The detection and elucidation of **1** and **2** were done by accurate mass HPLC-MS/MS on the basis of fragmentation pathways and by corroboration with authentic standards as detailed earlier.[28] The contents of **1** and **2** in the organic extracts of mycelia and broth, collected at periods of regular time intervals, were determined to provide an insight into the production kinetics as a function of time (Figure 3).

Both when illuminated and in the dark, the production of **2** commenced as early as 24 h (Figure 3a). The content of **2** gradually increased until 48 h, after which it remained almost the same, with some minor changes between 168 and 192 h. In order to obtain a smooth curve depicting the overall pattern production of **2**, we evaluated the moving averages, taking into account the values preceding and succeeding a particular point in time. The amount of **2** produced by the endophyte after fermentation for 9 days was comparable under both light and dark conditions. In conclusion, the accumulation of **2** in the cells started immediately irrespective of the conditions of illumination, and the concentration of **2** increased steeply in the first 24 h followed by a sharp decrease afterward until the end of 216 h (Figure 4a). Compound **2** was not detected in the spent medium (broth) under both light and dark
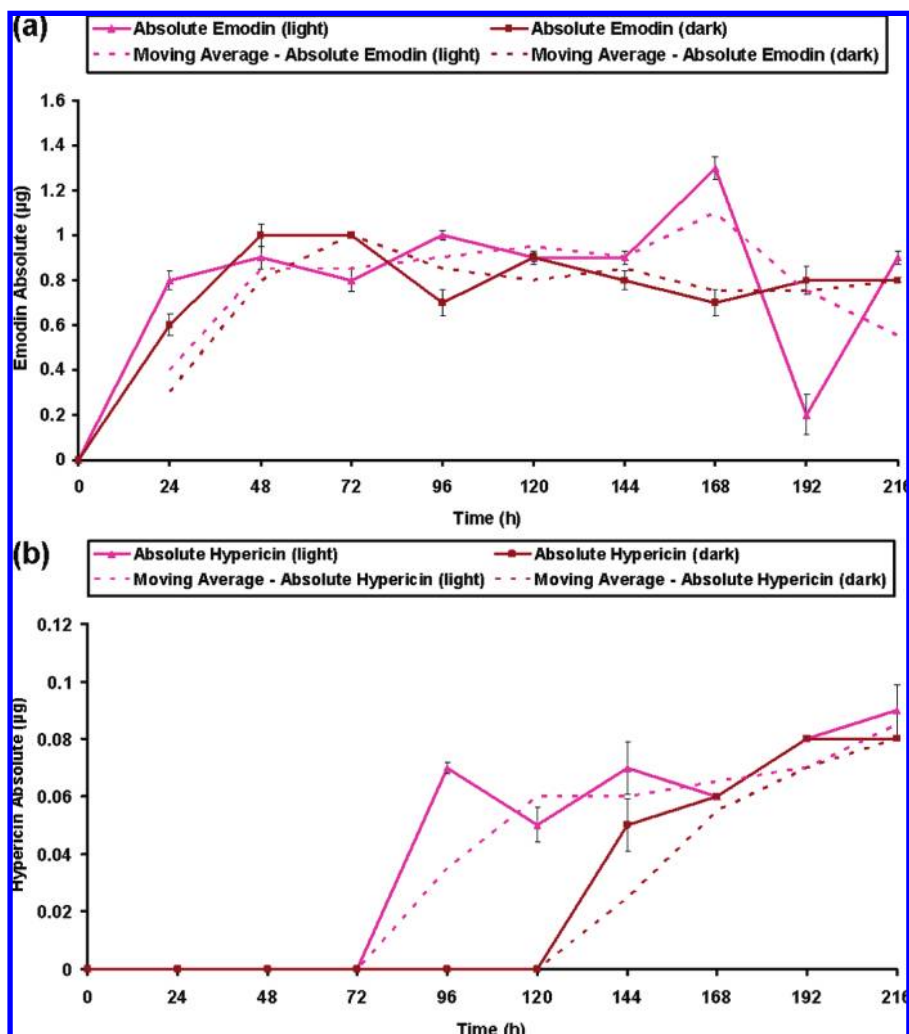
Figure 3. Production kinetics of the cultured endophyte producing hypericin (**1**) and emodin (**2**) under light and dark conditions at different time points of fermentation. (a) Emodin (**2**) production kinetics. (b) Hypericin (**1**) production kinetics.

conditions (LOD = 3.0 pg mL^{-1}), revealing that **2** was accumulated as intracellular metabolite without being released into the medium. Furthermore, compound **2** was not found at 0 h. Hence, **2** had not been carried over from the original plant material via the mycelia of the fungus, i.e., via the inoculum plugs.

The production of **1** did not start immediately in the light (Figure 3b). It could be detected only from 96 h of submerged fermentation onward, after which it gradually increased up to the end of the ninth day. Interestingly, however, the production of **1** commenced much later in darkness, only from 144 h onward (Figure 3b). Nevertheless, final concentrations and contents at 216 h were similar on illumination and in the darkness. It could, therefore, be concluded that although illumination was not a potent external factor in determining the microbial biosynthesis of **1**, it definitely had an effect, to some extent, on hastening the start of the biosynthesis. The evaluation of the intracellular concentrations of **1** (Figure 4b) revealed that in both light and dark conditions, there was a sharp increase in the postproduction initial concentrations for around 24 h, after which it remained constant and did not decrease, unlike compound **2**. As with **2**, **1** was not detected in the spent medium or at the start of the experiment (0 h) under conditions of either light or darkness.

The discovery of this endophyte producing both **1** and **2**[28] initially raised the question whether it was actually capable of producing both the metabolites or if compound **2** produced metabolically by the cultured endophyte underwent postmetabolic dimerization and oxidation to **1** through an intermediate **4** by visible

light irradiation. That would qualify compound **1** as a postmetabolic product of the endophyte. However, it has now become evident that light has no effect on the production of either **1** or **2** by the cultured endophyte, although it has some effect on the kinetics of production of **1**. Furthermore, there was no detectable production of compound **4** in any experiment. Hence, it is proved that both **1** and **2** are actual metabolic products that are accumulated by the endophyte.

Presence/Expression of the *hyp-1* Gene in the Endophyte. It was proposed that in the host plant cell cultures[22] direct and complex enzymatic conversion of **2** to **1** is governed by the product of the candidate *hyp-1* gene, the Hyp-1 phenolic coupling protein. This enzyme was considered to be responsible for several subsequent reactions leading to **1**. However, as we previously reported,[23] the transcript level of the respective gene in planta did not correspond with tissue-specific accumulation of **1**. In an attempt to isolate a homologous sequence from *T. subthermophila*, we tested the presence of the gene by PCR amplification with two pairs of *H. perforatum hyp-1* gene specific primers. No specific amplification product homologous to the *hyp-1* gene was amplified on either DNA or RNA/cDNA template from *T. subthermophila*. Distinct amplification product was obtained only by RT-PCR with a second set of primers (amplifying 570 bp long fragment on cDNA in *H. perforatum*). The amplified product was longer than in *H. perforatum* (Figure 5). Sequencing of this cDNA gene fragment did not reveal any nucleotide similarity with the published cDNA of the
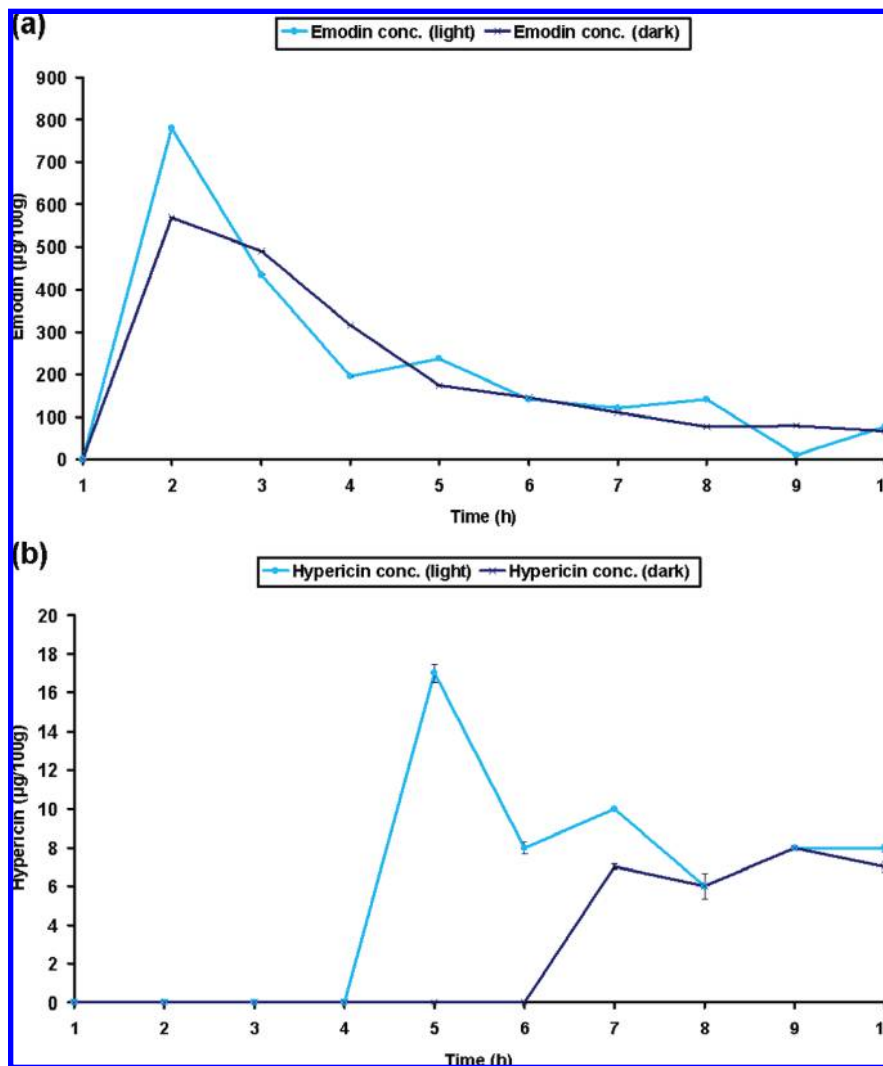
Figure 4. Intracellular concentration kinetics of the cultured endophyte under light and dark conditions at different time points of fermentation. (a) For emodin (**2**). (b) For hypericin (**1**).
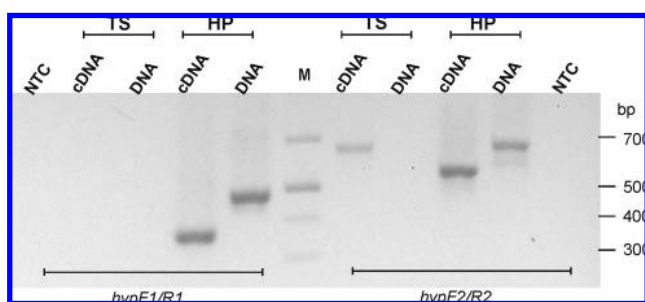
Figure 5. PCR/RT-PCR of the *hyp-1* gene by using two sets of *H. perforatum* specific primers (*hypF1/R1*, *hypF2/R2*). DNA or cDNA from *Thielavia subthermophila* (TS) and *H. perforatum* (HP) were used in the amplification reactions. NTC, no template control; M, DNA size marker.

hyp-1 gene from *H. perforatum*. The size difference between the *hyp-1* gene transcript and the genomic fragment amplified on DNA template from *H. perforatum* is due to the presence of intron within the gene sequence. The absence of the homologous sequence of the *hyp-1* gene in *T. subthermophila* indicates that if the *hyp-1* gene is involved in the biosynthesis of **1** in the host plant in the proposed manner,[22] then the biosynthetic pathway in the endophytic fungus might be different and/or governed by a different molecular mechanism than the host plant or host cell suspension cultures.

Hypericin (1) Biosynthesis by the Endophytic Fungus Is Not Light-Mediated. We postulate a new possibility concerning the final step(s) of the microbial pathway leading to **1** on the basis of the experimental results. Seemingly, both **1** and **2** could be products of oxidation and dimerization of the precursor **3**. This would mean that **1** and **2** are both secondary metabolites of the endophyte. Compound **2** would not then be the precursor of **1** that accumulates as an intermediate metabolite, and the pathway would not proceed via **4**. This is again emphasized by the lack of dependence of the production of **1** on light and the absence of **4** in all stages of culture. Therefore, it might be presumed that there could be some genetic factors responsible for the direct and specific conversion of **3** to **1**. This step would then also be light-independent. Earlier studies in plant systems revealed the independence of production of **1** from light, although the amounts varied.[29]

Perspectives on the Endophytic Hypericin (1) and Emodin (2) Biosynthesis in Relation to the Host Plant. The metabolomics of endophytes are dependent not only on the respective host plants but also on the ecosystem to which the host belongs.[30] The host preference and diversity of endophytes in various settings has been the subject of a plethora of investigations. A general consensus has been reached on the population pressure and diversity of endophytes in host plants growing in different ecosystems. Of particular interest has been the tropical environment; it has been postulated in general and shown in specific cases that tropical environments are the best suited setting for plant−microbe

interactions.[31−35] It has been hypothesized that the optimum setting for these interactions leads to horizontal gene transfers (HGT) or genetic recombinations, from the plant to its endophytic counterpart or vice versa, that lead to "novel" endophytes that are capable of acquiring the necessary cellular machinery for accumulating certain metabolites specific to the host plants themselves. Such gene transfer mechanisms have been anticipated for endophytes from other plant species, such as *Nothapodytes foetida (N. nimmoniana)*,[36,37] *Juniperus recurva*,[38] *Juniperus communis*,[39] *Camptotheca acuminata*,[40] and *Podophyllum peltatum*,[41] to name a few. The concept of HGT must not, however, be confused with that of the horizontal or vertical transmission of endophytic microorganisms. Nevertheless, with regard to endophytic microorganisms, one question that this hypothesis does not address is whether HGT and the site of detachment and/or integration in the host and/or recipient genome, respectively, is always a matter of "chance" or definitive. Added to this is the predicament about the expression of a gene or gene cluster in fungal systems acquired from plant systems. For example, in any attempt at isolating and bioprospecting endophytes for host metabolites, it has always been seen that out of a plethora of different endophytes isolated, only one or a few are capable of possessing the potential of indigenously accumulating host-specific metabolites; this of course could be rightly described as a "genetic serendipity" in the absence of evidence suggesting otherwise. Furthermore, the chance of HGT might be different in endophytic bacteria than in endophytic fungi. When assessing endophytic *T. subthermophila* isolate in terms of the above hypothesis, the results showed that the *hyp-1* gene was absent in the genome of the endophyte. Considering the fact that **2** might be the direct precursor of **1** in *Hypericum* plants, undergoing direct and specific conversion using the Hyp-1 protein [as was originally proposed by Bais et al.[22] in *H. perforatum* cell cultures grown in the dark (Figure 1c)], it is compelling that HGT might not be the only mechanism by virtue of which endophytes might produce compounds specific to their host plants. Therefore, in light of our current results and in addition to the current HGT hypothesis, we propose an alternative hypothesis. It is true that a particular environmental setting, like the tropical setting, greatly influences the species interactions (including plant−microbe and even microbe−microbe).[31−35] It is probable that this setting will favor the HGT mechanism, but there is a possibility that a particular endophyte could produce a host metabolite without any HGT. The concept of HGT, applied to endophytic microorganisms, centers on the idea that during the course of evolution an endophyte and a host started coexisting followed by some gene transfer "by chance" to provide it with a certain trait. However, considering the fact that fungi themselves are subject to evolution, it is highly feasible that during the coexistence of an endophyte with its host it underwent an independent evolution to develop its own molecular machinery (host−microbe coevolution). This could be due to "the same" selection pressure, viz., environmental stress, pathogen attack, insect attack, other factors, or a combination of these. This is partly in line with the recently proposed "xenohormesis" hypothesis,[42] although our subject is exclusively endophyte-specific. The tenets of our hypothesis may be summarized as follows: (a) the evolution of an endophytic fungus to intrinsically accumulate a metabolite that is essentially produced by its host and thus host-specific is subject to an "identical" selection pressure and organ-specific; (b) this potential is not serendipitous, but dependent on "both" the host and the microbe genotype-specific features such as genus, species, type of organ, and type of metabolites, simultaneously; and (c) suitable environmental factors such as tropical environments favor the increase in biodiversity and population of the endophytes, thereby exposing a larger number of endophytic traits to a particular in planta selection pressure. This concept may be called the "trait-specific endophytic infallibility" hypothesis based on the differential abilities of endophytes to indigenously accumulate specific me-
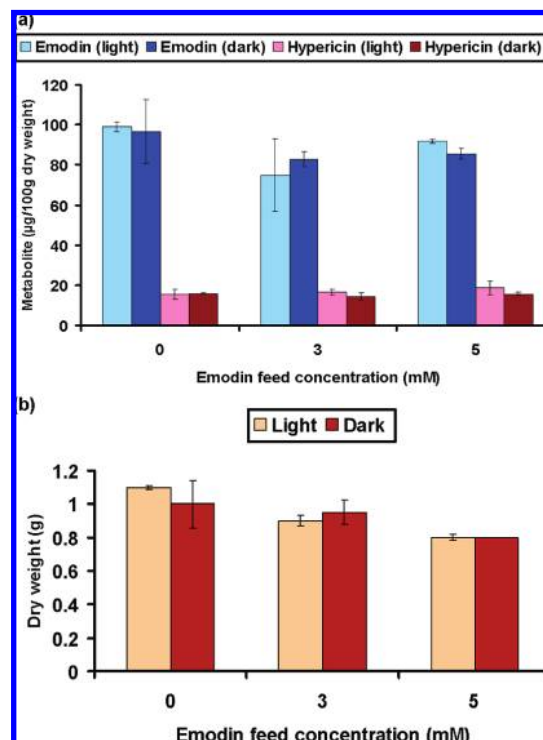
Figure 6. (a) Accumulation of hypericin (**1**) and emodin (**2**) by the cultured endophyte under submerged shake-flask conditions with additional feeds of various concentrations of emodin (**2**). (b) Effect of additional emodin (**2**) feeding on the growth of the cultured endophyte. All values represent after fermentation for 216 h.

tabolites based on their intrinsic traits subjected to specific selection pressures. Thus, this hypothesis could be used to explain the following: (a) difference in the metabolomics of an endophyte and its host; (b) difference in the metabolomics of an endophyte within its host and in axenic cultures, as also suggested by Strobel et al.[30] although in a different perspective; and (c) why only certain specific novel endophytes are capable of producing certain host-specific compounds. Admittedly, suitable experimental designs should be developed to test this hypothesis on a case-by-case basis in order to be able to verify or modify it.

Effect of Emodin (2) Spiking on Growth and Production. The endophytic fungus was spiked with different concentrations of **2** under submerged fermentation conditions, similar to those of the kinetics study, in order to study the effects of the additional spiking on the growth and production of **1** and **2** by the axenic endophyte in vitro. The spiking was performed under conditions of both light and darkness to determine the effect of light on the results. It was observed that the addition of **2** to the growth media did not stimulate or inhibit the production of either **1** or **2** by the cultured endophyte (Figure 6a). These remained unchanged even with or without the illumination conditions. Analyses of the spiked spent broths revealed that the endophyte did not take up **2** from the media. Interestingly, however, the addition of **2** had a negative impact on the growth of the endophyte both in the light and in darkness (Figure 6b). The growth was inversely proportional to the amount of **2** added and independent of the presence or absence of irradiation.

Regulation of Hypericin (1) and Emodin (2) Production in the Endophytic Fungus. Compound **1** is a peculiar metabolite that exhibits high cytotoxicity upon excitation by irradiation with visible light, a mechanism known as photodynamic activity.[10] It has been proposed that the localization, and probably the synthesis, of **1** in plants occurs in specialized structures called the dark glands.[43−45] Even though the current understanding of the ultrastructure of the dark glands does not provide sufficient evidence

concerning the in situ biosynthesis of **1** in plants, it is evident that the purpose of the localization of this photodynamic metabolite in the dark glands is to protect it from light and, hence, prevent autocytotoxicity. The endophytic fungus *T. subthermophila* does not contain any dark glands or other light-protection structures that are associated with its host plant. This raises the question about the regulation of production of **1** in the fungus so as to avoid self-damage. On one hand, it might be presumed that this particular endophyte has not developed an independent regulation mechanism for accumulating **1**. The explanation for this could be that this endophyte has been isolated from the inner stem tissues of the *H. perforatum* plant, where there is complete absence of light, thus protection from the photodynamic effects of **1** and **2**. This increasingly lends support to the mutual symbiotic association of the host and its endophytic counterpart, the former protecting the latter from light and the latter contributing to the chemical defense of the former. This, however, does not ratify the fact that there is no additional regulatory mechanism within the endophyte itself, since the endophyte continues to accumulate **1** and **2** under axenic conditions in vitro, even after separation from the host. It is well known that the metabolic regulation of an endophytic fungus might be substantially different from that which occurs inside the host plant.[30] In this connection, the concept of trait-specific endophytic infallibility is suitable. There is a high probability that this endophyte is able to produce **1** and **2** independently irrespective of the genes or pathways in its host, since (a) this production has been seen to be light-independent, (b) compound **4** has not been detected, and (c) the *hyp-1* gene has not been found in the endophyte genome. Therefore, the genetic programming of regulation of **1** production must lie in the gene(s) actually responsible for the final step in the microbial biosynthesis. Such an additional regulation mechanism has been insinuated earlier for *H. perforatum* cell cultures,[29] but its similarity, if any, to the microbial system could be confirmed only by further in planta investigation of the endophytic production of **1** and **2** at the molecular level.

Costs and Benefits of Endophytic Hypericin (1) and Emodin (2) Biosynthesis to the Host Plant. It is evident from the growth kinetics of the endophyte that light does not play any role in its growth. Considering the fact that this particular endophyte was isolated from the inner stem tissues of *H. perforatum*, where there is no possibility of illumination, it can be concluded that the survival and growth of the endophyte in the host is unaffected by the lack of light. Furthermore, the production pattern of both **1** and **2** by the cultured endophyte reveals that it might demonstrate similar or different metabolomics in the host, independent of light as a factor. Taking note of the fact that novel endophytes might serve as "acquired immune systems"[32] to their respective hosts, the intrinsic defense potential provided by the endophytes in vivo is currently a "variable" factor. This is because the potential of the low-biomass endophyte infections to manifest major chemical signatures in planta has to be assessed by inexplicably selective and sensitive measurements. Using the currently available experimental methodologies, it is almost impossible to detect the differences in metabolite amounts with and without endophyte infections. Clearly, this calls for alternative experimental designs that could address such sensitive changes in the metabolite spectra under in vivo conditions.

Furthermore, in *Hypericum* species, it has been found that **1** is produced by the plant as a mode of chemical defense against a variety of specific and nonspecific microbial pathogens and a number of insect pests.[46,47] Compound **1** is localized[43] and probably also synthesized in the dark glands,[44,45] which are dispersed over all above-ground parts of the plant (flowers, capsules, leaves) but not in the roots.[48] Additionally, the role of **1** in the *Hypericum* plant defense has been directly demonstrated by its increased production in response to stress. These include *Hypericum*-specific fungal pathogens,[49] nonspecific pathogens,[50] chemical elicitors such

as mannan[51] and jasmonic acid,[52] insect pests,[53] and mechanical stress such as cork pieces.[54] Therefore, the contribution by the endophytic *T. subthermophila* in producing **1** and **2** as part of the defensive mechanism of its host *H. perforatum* could be a practical possibility. Thus, it may be permissible to presume that this endophyte could not only contribute to the metabolites in the host tissues but also play an important role in the chemical defense of the host. Admittedly, this particular endophyte was isolated from the stems of the host and not from the leaves and, like many other localized endophytes, is not expected to provide "systemic" defense to the host plant. On the basis of these facts, it may be assumed that in the leaves and flower parts the high number of dark glands, and consequently high amounts of **1**, is sufficient to provide localized leaf/flower protection, and this endophyte might be only helping in localized stem protection. This is emphasized by the fact that the endophyte does not release **1** or **2** in the broth in axenic cultures in vitro; thus, the same could be expected under in vivo conditions, the metabolites not being released into the plant sap.

Correlation between Growth and Production in the Endophytic Isolate. Even though a number of novel endophytes, capable of indigenously producing various important metabolites, have been isolated and characterized to date, only a few workers have addressed the correlations between the growth and metabolomics of the endophytes in axenic cultures. For instance, Li et al.[55] showed that successive cultures of the endophytic fungus *Periconia* sp., isolated from *Torreya grandifolia*, resulted in the attenuation of Taxol production, although the fungal growth itself was unaffected. Again, we have recently demonstrated an inverse relation between the growth and the production of camptothecin over successive generations by an endophytic *Fusarium solani* isolated from *Camptotheca acuminata*.[40] With regard to endophytic *T. subthermophila*, it has been revealed that the addition of **2** to the submerged cultures resulted in the suppression of growth (Figure 6b). This lends support to the fact that the growth of the endophyte is inversely related to the production of **2** in axenic cultures. Alternatively, it might also be putatively inferred that there is in fact an additional regulatory mechanism that controls the subcellular trafficking of **2** in the endophyte, although not very similar, from the perspective of the *hyp-1* gene, to what was proposed in the *H. perforatum* cell cultures.[29] The production of **1** and **2** by cultured *T. subthermophila* we report in this paper is less than what we initially observed.[28] This reveals a similar pattern of attenuation that could also be observed for endophytic *F. solani*.[40] Further studies on the production pattern over generations and on storage under different conditions are underway.
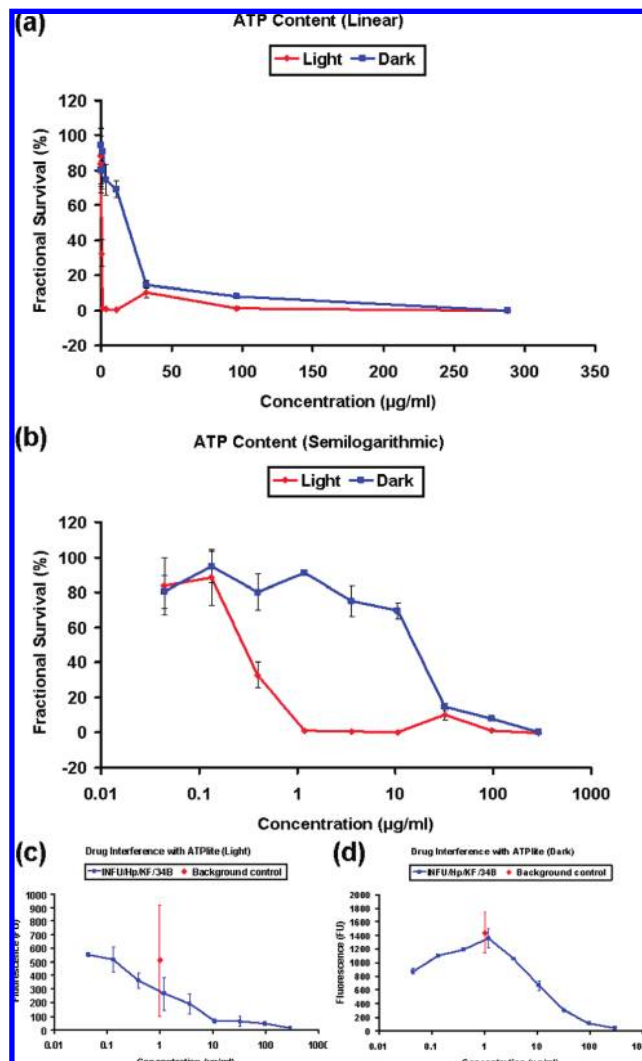
Cytotoxic and Photodynamic Efficacies of the Fungal Metabolites. In order to investigate the exploratory in vitro cytotoxic effects of photoactivated fungal extract containing **1** and **2** metabolized by the fungus, the human acute monocytic leukemia cell line (THP-1) was incubated in parallel with the various concentrations of the fungal extract (Figure S4, Supporting Information) in the dark and after light activation, respectively. Subsequently, the cytotoxicity was assessed using the resazurin-based assay to measure the THP-1 mitochondrial succinoxidase inhibition (Figure 7), as well as by using the ATPlite assay to measure the THP-1 cytoplasmic ATP depletion (Figure 8). The cytotoxic effect of the fungal extract was found under both dark and light-activated conditions. In both conditions, a concentration-dependent cytotoxicity was observed. However, the cytotoxicity of the fungal extract was much more pronounced after irradiation with light for 20 min in both the assay types, revealing the photodynamic properties of the fungal metabolites. The resazurin assay revealed the greatest viability gap at a concentration of 1.185 μg mL^{-1} [92.7 (dark) vs 4.9% (light)] (Figure 7b). Similarly, the ATPlite assay also revealed the greatest viability gap at the same concentration [91.1 (dark) vs 1.0% (light)] (Figure 8b). It is imperative from these results that some form of intrinsic regulation
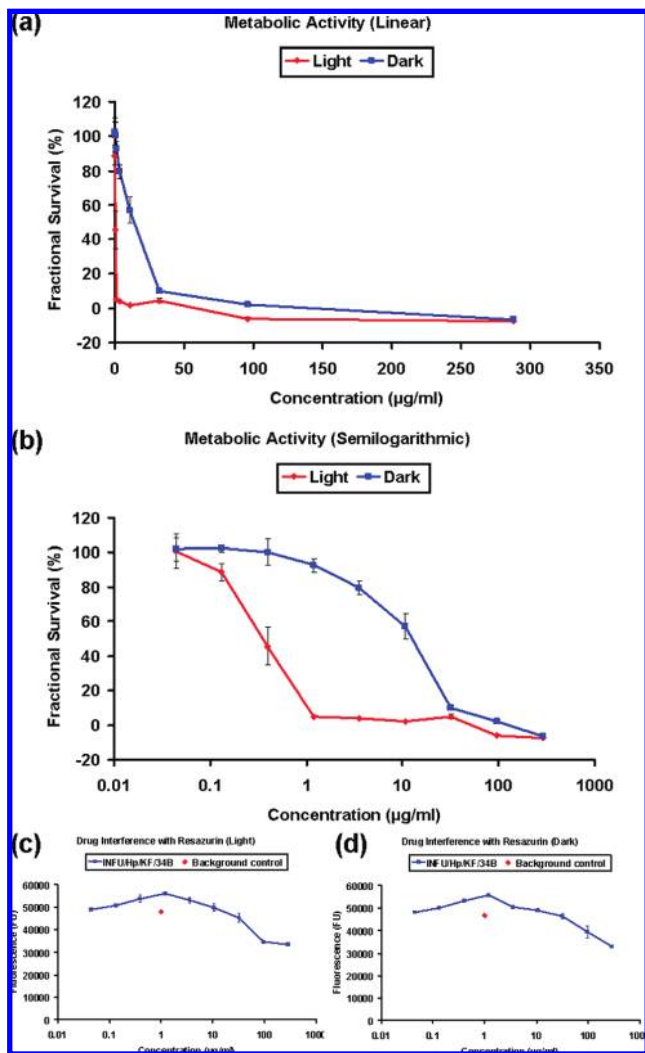
Figure 7. Resazurin-based in vitro cytotoxic assay of the fungal extract against THP-1 cells under light protection and after light activation. (a) Linear representation of fractional survival (FS) of THP-1 as a function of concentration. (b) Semilogarithmic representation of the FS of THP-1 as a function of concentration. (c) Interference of the sample with the indicator (resazurin) in light conditions. (d) Interference of the sample with the indicator (resazurin) in dark conditions.

Figure 8. In vitro cytotoxic assay of the fungal extract against THP-1 cells using ATPlite under light protection and after light activation. (a) Linear representation of fractional survival (FS) of THP-1 as a function of concentration. (b) Semilogarithmic representation of the FS of THP-1 as a function of concentration. (c) Interference of the sample with the indicator (ATPlite) in light conditions. (d) Interference of the sample with the indicator (ATPlite) in dark conditions.

mechanism is required to protect the fungus from autocytotoxicity, because the cytotoxic effects of fungal **1** and **2** were enhanced in the presence of light and because the fungus still continues to accumulate these metabolites in axenic cultures outside its host and also under light.

Effect of Fungal Metabolites on Morphology of Human Cancer Cell Line THP-1. In order to ascertain the effect of the fungal metabolites on the THP-1 morphology under dark and photoactivated states, microscopic studies of untreated and treated cells were performed (Figure S5, Supporting Information). The untreated cells were round, single cells in suspension, and some cells were in clusters. The treated cells showed reduction in size and condensation of the nucleus, and the protoplasmic extensions were reduced. The cells treated with photoactivated metabolites showed even more visible apoptosis with drastic condensation of the cytoplasm and nucleus and marginalization of the chromatin material in the nuclei (Figure S5b, Supporting Information). This, yet again, revealed the high photodynamic properties of the fungal metabolites that would necessitate some form of regulation of endophytic production of these metabolites.

Experimental Section

Isolation and Identification of the Endophytic Fungus. As part of an effort to identify endophytic fungi that produce **1**, wild specimens of *H. perforatum* were collected at the bloom stage from natural populations at Harwan, Jammu and Kashmir, India. The detailed procedure for the isolation of endophytic fungi has been reported earlier.[28] Among all the isolated putative endophytes, only one was able to produce emodin (**2**) and hypericin (**1**) under axenic conditions in vitro (coded INFU/Hp/KF/34B)[28] and was taken up for further studies. The molecular analysis of the fungus based on the 28S large subunit (LSU) rRNA gene was inconclusive; hence we resorted to identification by ITS analysis (ITS1 and ITS2 regions and the intervening 5.8S rDNA region). The endophytic fungus was cultured on PDA (potato dextrose agar; DIFCO, cat. no. 213400) for 5 days at 28 ± 2 °C. The mycelium was scraped directly from the surface of the agar culture (5 days old) and weighed. Nucleic acids were extracted and purified using the AppliChem DNA isolation kit for genomic DNA (AppliChem GmbH, Darmstadt, Germany) using the Chomczynski method,[56] suitably modified. For identification and differentiation, the internal transcribed spacer regions (ITS1 and ITS2) and the intervening 5.8S rRNA region were amplified and sequenced[57] 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 GC-3′).[46] 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). 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.

Maintenance and Storage of the Endophytic Fungus. The axenic culture, accumulating **1** and **2**, was coded as INFU/Hp/KF/34B and was routinely maintained on PDA, SA (Sabouraud dextrose agar; DIFCO, cat. no. 210950), and CDA (Czapek-Dox Agar; Merck, Darmstadt, Germany) in active form. For long-term storage, the colonies were preserved in the vegetative form in 15% (v/v) glycerol at −70 °C. Agar blocks impregnated with mycelia were used directly for storage of the vegetative forms. Furthermore, the endophytic fungus has been deposited at the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ), Braunschweig, Germany (accession number DSM 21024).

Morphological Studies of the Endophytic Fungus. The endophytic fungus, INFU/Hp/KF/34B, growing on PDA, SA, and CDA was examined after 2, 3, 4, 5, and 10 days to study the macroscopic morphology. Hyphae from the agar plate were aseptically transferred to slides for microscopy. A Leica DM-R light microscope (Leica Microsystems GmbH, Wetzlar, Germany) was used to examine the microscopic features of the fungus.

Establishment of Hypericin (1) and Emodin (2) Production as a Function of Time. The endophyte was cultured in PDB (potato dextrose broth; DIFCO, cat. no. 254920). A set of 10 conical flasks (500 mL) was used, each with four indentations and containing 100 mL of PDB, adjusted to pH 5.6 before autoclaving. The fungus was inoculated into each flask from the parent axenic culture. These flasks were incubated at 28 ± 2 °C with shaking (200 rpm) on a rotary shaker (Heidolph UNIMAX 2010, Germany). Each flask represented one time point for termination of fermentation followed by extraction and analysis for determination of the production of **1** and **2**. The first sample was taken after 2 h of inoculation (0 h), and subsequently the other samples were taken after every 24 h, up to 216 h. For each sample, the dry weight of biomass was determined after termination of fermentation to the desired time point. A similar set of 10 flasks was prepared simultaneously and processed in parallel in the same way. The only difference was that the entire procedure was performed under complete light protection, from inoculation through fermentation to extraction and analysis of **1** and **2**. Three replicates of each experiment set were undertaken to get reproducible data.

Emodin (2) Spiking under Submerged Fermentation Conditions. The spiking experiments were performed in a similar fashion to the fermentation for kinetic studies. Groups of three conical flasks of 500 mL capacity were used, each with four indentations and containing 100 mL of PDB adjusted to pH 5.6 before autoclaving. The fungus was inoculated into each flask from the parent axenic culture. These flasks were incubated at 28 ± 2 °C with shaking (200 rpm) on a rotary shaker for 216 h. The first set was the control and was not fed with **2**. In the second and third sets, 3 and 5 mM concentrations of **2**, respectively, were fed before commencing with the shake-flask fermentation. A similar setup of three sets was prepared simultaneously and processed in parallel under complete light protection from inoculation through fermentation to extraction and analysis of **1** and **2**. For each sample, the dry weight of biomass was determined after termination of fermentation. Three replicates of each experiment set were undertaken to get reproducible data.

Preparation of Cell-Free Extract. The cell-free extract was prepared by filtering the incubated culture grown in PDB through muslin cloth under vacuum. The mycelia and broth were treated separately. The mycelial pellet was dried in an oven at ≤30 °C and the dry weight determined. The dried mycelial pellet was resuspended in deionized H$_2$O and sonicated in an ultrasonicator (Branson B-12, Danbury, Connecticut) at ≤4 °C. The milky fluid obtained was extracted with EtOH (3 × 50 mL), followed by extraction with CHCl$_3$−MeOH (4:1

v/v, 3 × 50 mL). The organic solvent was removed by rotary evaporation in vacuo at 30 °C. The dry organic extract was resuspended in 1 mL of HPLC grade MeOH. The spent broth (100 mL) was extracted three times in a similar fashion. The organic solvent was removed by rotary evaporation in vacuo at 30 °C, to obtain the extracellular organic extract. The final extracellular organic extract was resuspended in 1 mL of HPLC grade MeOH.

Quantitation of Hypericin (1) and Emodin (2). Quantitation of **1** and **2** was performed according to Kusari et al.[28] by using a Thermo Finnigan Surveyor HPLC system (Ringoes, NJ) consisting of a Surveyor MS-pump and a Surveyor Autosampler-Plus (injection volume 5 μL) (Thermo Scientific). The compounds were separated on a Luna C$_{18}$ (50 × 3 mm, 3 μm particle size) column from Phenomenex (Torrance, CA). The mobile phase consisted of H$_2$O containing 10 mM NH$_4$OAc (pH 5.0) (A) and MeCN−MeOH, 9:1 (B). Samples were separated using a gradient program as follows: (flow rate of 250 μL min^{-1}) 55% A isocratic for 2 min, linear gradient to 100% B over 6 min (flow rate of 300 μL min^{-1}). After 100% B isocratic for 7 min, the system was returned to its initial conditions (55% A) within 1 min and was equilibrated for 4 min before the next run was started. MS detection (multiple reaction monitoring mode) was performed by using a TSQ Quantum Ultra AM (Thermo Scientific) equipped with an ESI ion source (Ion Max) operating in negative mode. N$_2$ was employed as both the sheath (50 arbitrary units) and auxiliary (8 arbitrary units) gas, and Ar served as the collision gas with a pressure of 1.5 mTorr. The capillary temperature was set to 250 °C. External calibration was performed in the range 0.01−10.0 μg mL^{-1} for **1** and 0.005−10.0 μg mL^{-1} for **2**. Correlation coefficients for the linear calibration curves were >0.995 for both compounds.

HRMS Screening for Emodin Anthrone (3) and Protohypericin (4). Compounds **1** and **2** were identified by HRMS fragment spectra (LTQ-Orbitrap spectrometer, Thermo Scientific), which were consistent with the authentic standards (**1** from Sigma-Aldrich Chemie GmbH, Steinheim, Germany, and **2** from AppliChem GmbH). Additional screening for **3** and **4** was performed in full scan negative mode. The spectrometer was equipped with a Thermo Surveyor system consisting of a LC-pump and autosampler (injection volume 5 μL). N$_2$ was used as sheath gas (5 arbitrary units), and He served as the collision gas. The separations were performed by using a Phenomenex Synergi Fusion RP column (4 μm, 2 × 150 mm) with a H$_2$O (+0.1% HCOOH, +10 mM NH$_4$OAc) (A)/MeCN (+0.1% HCOOH) (B) gradient (flow rate 0.25 mL min^{-1}). Samples were analyzed by using a gradient program as follows: 50% A isocratic for 2 min, linear gradient to 100% B over 8 min; after 100% B isocratic for 48 min, the system was returned to its initial condition (50% A) within 1 min and was equilibrated for 6 min. The spectrometer was operated in negative mode (1 spectrum s^{-1}; mass range 200−1000) with 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.

Detection of the *hyp-1* Gene in the Endophyte. Nucleic acids for detection of the presence and expression of the *hyp-1* gene in the endophyte by PCR and RT-PCR, respectively, were isolated using DNeasy- and RNeasy-Plant Mini Kits (Qiagen, Valencia, CA). The *hyp-1* gene-specific primers were designed on the basis of the published full-length cDNA of *hyp-1* from *H. perforatum* (accession number AY148090). Two sets of primers amplifying 368 bp (hyp-for1 5′-AGGCTGTTTAAGGCATTGGTCC-3′, hyp-rev1 5′-GCTTTCTTTTC-CCCGATCTTGAC-3′) and 570 bp long gene fragments (hyp-for2 5′-TTTCTGAATATGGCGGCGTACAC-3′, hyp-rev2 5′-CAAGCATCG-CAAAACACAAGACC-3′) were used. The expected lengths of the PCR products were based on the published *hyp-1* cDNA from *H. perforatum*. The binding sites of the PCR primers were localized inside the translated region of the gene (hyp-for1/rev1) or covered the whole translated region of the *hyp-1* gene (hyp-for2/rev2). Approximately 500 ng of total RNA was reverse transcribed by 10 mM anchored oligo-T primer and 200 U M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. DNA and cDNA from *H. perforatum* were used as positive controls of the PCRs. The amplification reactions were performed in 30 μL reaction volume containing 1× diluted *Taq*-polymerase reaction buffer with 1.5 mM MgCl$_2$ (Finnzymes, Espoo, Finland), 1.0 U DyNAzyme II DNA polymerase (Finnzymes), 0.2 mM dNTP (Finnzymes), 0.5 μM forward and reverse primer, and 50 ng of DNA or reverse transcribed RNA (cDNA). The reaction conditions were as follows: 95 °C for 3 min, 30 cycles (94 °C for 30 s, 58 °C for 30 s,

and 72 °C for 30 s), and 74 °C for 4 min, in MJ-Mini thermocycler (BioRad, Hercules, CA). Initially, gradient PCR (annealing from 52 to 60 °C) was applied to obtain distinct and specific PCR products. The length of the desired amplification product was verified by electrophoresis in 2% agarose gel dyed with GoldView (0.005% v/v; SBS Clontech, Beijing, China). The distinct PCR products from the endophyte were purified by Wizard SV gel and PCR Clean-Up System (Promega, Madison, WI) and directly sequenced with the forward and reverse primer. The assembled nucleotide sequences were aligned and compared with the publicly available database of DNA sequences (GenBank) by BLASTn to verify the nucleotide similarity with the *hyp-1* gene.

Culturing of the THP-1 Cell Line. The human acute monocytic leukemia cell line (THP-1), bearing DSMZ number ACC 16, was used. The THP-1 cells were grown in tissue culture flasks in complete growth medium in an atmosphere of 5% CO_2 and 90% relative humidity in a carbon dioxide incubator. The complete growth medium was prepared by using RPMI-1640 supplemented with 2 mM L-glutamine, 10% FBS, and penicillin (100 IU mL^{-1}, just before use) in double-distilled water. The pH of the medium was adjusted to 7.2, and the medium was sterilized by filtering through 0.2 μm filters in a laminar air flow hood under aseptic conditions.

Subculturing of the THP-1 Cell Line. For subculturing, the medium of the flask having subconfluent growth was changed 1 day in advance. The entire medium from the flask was taken out and discarded. Cells were washed with PBS. Then 0.5 mL of Trypsin-EDTA in PBS (prewarmed at 37 °C) was added to make a thin layer on the monolayer of the THP-1 cells. The flask was incubated for approximately 5 min at 37 °C and observed under a microscope. If the cells were found to be detached, complete growth medium (1 mL, prewarmed at 37 °C) was added to make the cell suspension. An aliquot was taken out and cells were counted and checked for viability with Trypan blue. Cell stock of more than 98% cell viability was accepted for determination of the in vitro cytotoxicity. The cell density was adjusted to 5.0×10^4 cells mL^{-1} by addition of more complete growth medium.

Preparation of Test Materials. The stock solution for the cytotoxicity assays was prepared by pooling together all the fungal extracts obtained from multiple fermentations and dissolving in DMSO. A final concentration of 7.2 mg mL^{-1} (filter-sterilized through 0.2 μm filter under vacuum after dissolving) could be achieved with the pooled extracts. From the stock solution, working concentrations were prepared such that the maximum concentration was 288.0 μL mL^{-1} and minimum concentration was 0.044 μL mL^{-1}, with a dilution factor (DF) of 3. Since the relative concentrations of **1** and **2** in the pooled fungal extract were unknown, the working solutions were prepared in μL mL^{-1} rather than in μM.

Cytotoxic Assay. The in vitro cytotoxicity against the human cancer cell line THP-1 was determined using 96-well flat bottom tissue culture plates (Corning B. V. Life Sciences, The Netherlands), with the parameters as shown in Figure S4 (Supporting Information). The aliquots of 100 μL of cell suspension (5.0×10^4 cells mL^{-1}) were added to each well and incubated for 24 h at 37 °C. The samples (100 μL in each well) of the desired concentrations were added under strictly subdued light conditions to the wells, giving a final well-volume of 200 μL and final cell concentration of 2.5×10^4 cells mL^{-1}. The cells were allowed to grow in the presence of different sample concentrations for another 48 h at 37 °C in the complete absence of light. In order to estimate the photodynamic cytotoxicity of the samples, a similar set was prepared in parallel and run simultaneously as follows: the plates containing the cells and the samples were exposed to light for 20 min before incubation, by placing them on 3 mm transparent plastic slides 7 cm above a set of three 20 W halogen lamps. The plates were constantly moved to avoid shadows. During irradiations, the temperatures never exceeded 32 °C. All experimental sets were repeated six times to obtain reproducible data. Three sets of controls were used, in parallel, in light and dark conditions, to validate the experimental data. The first control was the negative control, which consisted of media only, in six replicates. The second control was the background control in eight replicates. A third additional control was designed to test the sample–indicator interaction and interference, at all concentrations, and in duplicates. Two different methods were used in all cases in parallel to quantify the viable cells using a VICTOR X3 multilabel plate reader (PerkinElmer Life And Analytical Sciences, Inc., Boston, MA). The first method consisted of quantification using Resazurin (Sigma-Aldrich Chemie GmbH), to measure the mitochondrial activity. The second

method consisted of quantifying using ATPlite (PerkinElmer Life and Analytical Sciences, Inc.), to measure the available ATP concentration. The final relative viabilities were calculated and represented in percent fractional survival (FS).

Morphological Changes of Human Cancer Cell Line THP-1 on Treatment with Fungal Metabolites. The THP-1 cells were centrifuged at 2000 rpm for 10 min, spread over sterile glass slides, and allowed to air-dry under strict aseptic conditions. The air-dried smears were fixed in absolute MeOH for 2 min and stained with Giemsa (Sigma-Aldrich Chemie GmbH). These were subjected to microscopic studies using a Leica DM-R light microscope (Leica Microsystems GmbH) and photographed using the built-in digital camera. In order to ascertain the action mechanism of the fungal metabolites on THP-1, microscopic studies were performed with treated cells at all concentrations, for both the light-protected and light-activated assays in parallel.

Supporting Information Available: The general mechanism of the theoretical polyketide pathway with special emphasis on hypericin (**1**), the representative microscopic view of the endophytic *T. subthermophila*, as well as the ITS-5.8S rDNA sequence and the dendrogram showing its phylogenetic position; the diagrammatic layout of the 96-well plates representing the parameters of the cytotoxic assays using both the resazurin and ATPlite methods, and representative microscopic pictures depicting the morphology of the untreated THP-1 cells as well as those treated with light-activated fungal metabolites and treated with fungal metabolites not photoactivated. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Yazaki, K.; Okada, T. In *Medicinal and Aromatic Plants VI– Biotechnology in Agriculture and Forestry*; Bajaj, Y. P. S., Ed.; Springer-Verlag: Berlin, Germany, 1994; Vol. 26, pp 167–178.
(2) Nahrstedt, A.; Butterweck, V. *Pharmacopsychiatry* **1997**, *30*, 129–134.
(3) Hickey, M.; King, C. *100 Families of Flowering Plants*, 2nd ed.; Cambridge University Press: Cambridge, UK, 1981.
(4) Wichtl, M. *Z. Phytother.* **1986**, *3*, 87–90.
(5) Raffa, R. B. *Life Sci.* **1998**, *62*, 265–270.
(6) Zaichikova, S. G.; Grinkevich, N. I.; Barabanov, E. I. *Farmatsiya* **1985**, *34*, 62–64.
(7) Radulovic, N.; Stankov-Jovanovic, V.; Stojanovic, G.; Smelcerovic, A.; Spiteller, M.; Asakawa, Y. *Food Chem.* **2007**, *103*, 15–21.
(8) Razinkov, S. P.; Yerofeyeva, L. N.; Khovrina, M. P.; Lazarev, A. I. *Zh. Ushn. Nos. Gorl. Bolezn.* **1989**, *49*, 43–46.
(9) Martinez, B.; Kasper, S.; Ruhrmann, S.; Moller, H. J. *Nervenheilkunde* **1993**, *36*, 103–108.
(10) Kubin, A.; Wierrani, F.; Burner, U.; Alth, G.; Grunberger, W. *Curr. Pharm. Des.* **2005**, *11*, 233–253.
(11) Delaey, E. M.; Obermüeller, R.; Zupko, I.; de Vos, D.; Falk, H.; de Witte, P. A. *Photochem. Photobiol.* **2001**, *74*, 164–171.
(12) Hadjur, C.; Richard, M. J.; Parat, M. O.; Jardon, P.; Favier, A. *Photochem. Photobiol.* **1996**, *64*, 375–381.
(13) Kamuhabwa, A. R.; Agostinis, P. M.; D'Hallewin, M. A.; Baert, L.; de Witte, P. A. *Photochem. Photobiol.* **2001**, *74*, 126–132.
(14) Brockmann, H.; Haschad, M. N.; Maier, K.; Pohl, F. *Naturwissenschaften* **1939**, *32*, 550–550.

(15) Brockmann, H.; Pohl, F.; Maier, K.; Haschad, M. N. *Ann. Chem.* **1942**, *553*, 1–52.

(16) Brockmann, H.; Falkenhausen, E. H.; Dorlares, A. *Naturwissenschaften* **1950**, *37*, 540–540.

(17) Thomson, R. H. *Naturally Occurring Quinones*; Butterworth's Scientific Publications: London, UK, 1957.

(18) Falk, H. *Angew Chem. Int. Ed.* **1999**, *38*, 3116–3136.

(19) Zobayed, S. M. A.; Afreen, F.; Goto, E.; Kozai, T. *Ann. Bot.* **2006**, *98*, 793–804.

(20) Karppinen, K.; Hokkanen, J.; Mattila, S.; Neubauer, P.; Hohtola, A. *FEBS J.* **2008**, *275*, 4329–4342.

(21) Chen, Z. G.; Fujii, I.; Ebizuka, Y.; Sankawa, U. *Phytochemistry* **1995**, *38*, 299–305.

(22) Bais, H. P.; Vepachedu, R.; Lawrence, C. B.; Stermitz, F. R.; Vivanco, J. M. *J. Biol. Chem.* **2003**, *278*, 32413–32422.

(23) Kosuth, J.; Katkovcinova, Z.; Olexova, P.; Cellarova, E. *Plant Cell. Rep.* **2007**, *26*, 211–217.

(24) Falk, H.; Meyer, J.; Oberreiter, M. *Monatsh. Chem.* **1993**, *124*, 339–341.

(25) Brockmann, H.; Kluge, F.; Muxfeldt, H. *Chem. Ber.* **1957**, *90*, 2302–2318.

(26) Mazur, Y.; Bock, H.; Lavie, D. U.S. Patent 5,120,412, 1992.

(27) Falk, H.; Schoppel, G. *Monatsh. Chem.* **1991**, *122*, 739–744.

(28) Kusari, S.; Lamshöft, M.; Zühlke, S.; Spiteller, M. *J. Nat. Prod.* **2008**, *71*, 159–162.

(29) Bais, H. P.; Walker, T. S.; McGrew, J. J.; Vivanco, J. M. *In Vitro Cell. Dev. Biol.−Plant* **2002**, *38*, 58–65.

(30) Strobel, G. A.; Daisy, B.; Castillo, U.; Harper, J. *J. Nat. Prod.* **2004**, *67*, 257–268.

(31) Arnold, A. E.; Maynard, Z.; Gilbert, G. S.; Coley, P. D.; Kursar, T. A. *Ecol. Lett.* **2000**, *3*, 267–274.

(32) Arnold, A. E.; Mejía, L. C.; Kyllo, D.; Rojas, E. I.; Maynard, Z.; Robbins, N.; Herre, E. A. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15649–15654.

(33) Arnold, A. E. In *Current Trends in Mycological Research*; Deshmukh, D., Ed.; Oxford & IBH Publishing Co. Pvt. Ltd.: New Delhi, India, 2005; pp 49−68.

(34) Arnold, A. E. In *Tropical Forest Community Ecology*; Schnitzer, S. A.; Carson, W. P., Eds.; Wiley-Blackwell Scientific, Inc.: UK, 2008; pp 254−271.

(35) Rodriguez, R. J.; White, J. F., Jr.; Arnold, A. E.; Redman, R. S. *New Phytol.* **2009**, *182*, 314–330.

(36) Puri, S. C.; Verma, V.; Amna, T.; Qazi, G. N.; Spiteller, M. *J. Nat. Prod.* **2005**, *68*, 1717–1719.

(37) 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.

(38) Kour, A.; Shawl, A. S.; Rehman, S.; Sultan, P.; Qazi, P. H.; Suden, P.; Khajuria, R. K.; Verma, V. *World J. Microbiol. Biotechnol.* **2008**, *24*, 1115–1121.

(39) Kusari, S.; Lamshöft, M.; Spiteller, M. *J. Appl. Microbiol.* **2009**, *107*, 1019–1030.

(40) Kusari, S.; Zühlke, S.; Spiteller, M. *J. Nat. Prod.* **2009**, *72*, 2–7.

(41) Eyberger, A. L.; Dondapati, R.; Porter, J. R. *J. Nat. Prod.* **2006**, *69*, 1121–1124.

(42) Howitz, K. T.; Sinclair, D. A. *Cell* **2008**, *133*, 387–391.

(43) Briskin, D. P.; Leroy, A.; Gawienowski, M. *Plant Physiol. Biochem.* **2000**, *38*, 413–420.

(44) Cellarova, E.; Daxnerova, Z.; Kimakova, K.; Haluskova, J. *Acta Biotechnol.* **1994**, *14*, 267–274.

(45) Onelli, E.; Rivetta, A.; Giorgi, A.; Bignami, M.; Cocucci, M.; Patrignani, G. *Flora* **2002**, *197*, 92–102.

(46) Fields, P. G.; Arnason, J. T.; Fulcher, R. G. *Can. J. Bot.* **1990**, *68*, 1166–1170.

(47) Guillet, G.; Podeszfinski, C.; Regnault-Roger, C.; Arnason, J. T.; Philogene, B. J. R. *Environ. Entomol.* **2000**, *29*, 135–139.

(48) Hölzl, J.; Petersen, M. In *(Medicinal and Aromatic Plants−Industrial Profiles) Hypericum: The Genus Hypericum*; Ernst, E., Ed.; Taylor and Francis: London, UK, 2003; Vol. 31, pp 77−93.

(49) Cirak, C.; Aksoy, H. M.; Ayan, A. K.; Saglam, B.; Kevseroglu, K. *Plant Protect. Sci.* **2005**, *41*, 109–114.

(50) Sirvent, T. M.; Gibson, D. M. *Physiol. Mol. Plant Pathol.* **2002**, *60*, 311–320.

(51) Kirakosyan, A.; Hayashi, H.; Inoue, K.; Charchoglyan, A.; Vardapetyan, H. *Phytochemistry* **2000**, *53*, 345–348.

(52) Walker, T. S.; Bais, H. P.; Vivanco, J. M. *Phytochemistry* **2002**, *60*, 289–293.

(53) Sirvent, T. M.; Stuart, B.; Gibson, D. M. *J. Chem. Ecol.* **2003**, *29*, 2667–2681.

(54) Kirakosyan, A.; Hayashi, H.; Inoue, K.; Charchoglyan, A.; Vardapetyan, H.; Yammoto, H. *Russ. J. Plant Physiol.* **2001**, *48*, 816–819.

(55) Li, J. Y.; Sidhu, R. S.; Ford, E. J.; Long, D. M.; Hess, W. M.; Strobel, G. A. *J. Ind. Microbiol. Biotechnol.* **1998**, *20*, 259–264.

(56) Chomczynski, P.; Sacchi, N. *Anal. Biochem.* **1987**, *162*, 156–159.

(57) White, T. J.; Bruns, T.; Lee, S.; Taylor, J. W. In *PCR Protocols: a Guide to Methods and Applications*; Innis, M. A.; Gelfand, D. H.; Sninsky, J. J.; White, T. J., Eds.; Academic Press: San Diego, 1990; pp 315−322.