Flavonolignans from Aspergillus iizukae, a Fungal Endophyte of Milk Thistle (Silybum marianum)

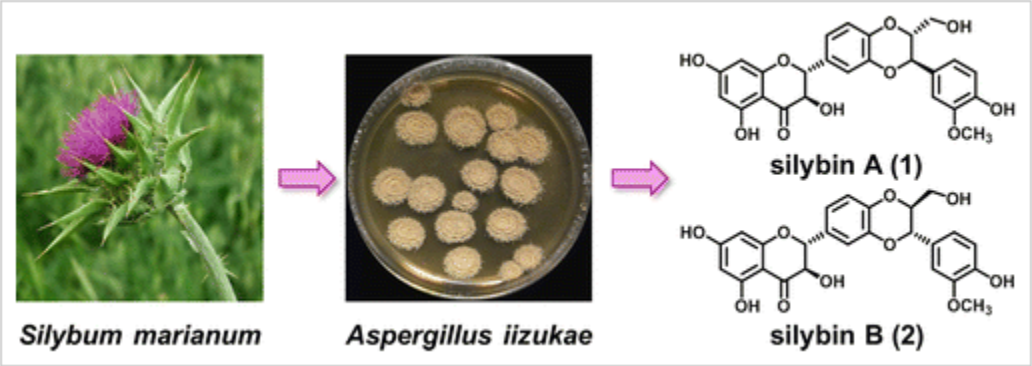
By: Tamam El-Elimat, Huzefa A. Raja, Tyler N. Graf, Stanley H. Faeth, Nadja B. Cech, and Nicholas H. Oberlies

El-Elimat T., Raja H.A., Graf T.N., Faeth S.H., Cech N.B., and Oberlies N.H. 2014. Flavonolignans from Aspergillus iizukae, a Fungal Endophyte of Milk Thistle (Silybum marianum). Journal of Natural Products 77: 193–199.

Made available courtesy of American Chemical Society:
http://dx.doi.org/10.1021/np400955q

Abstract:

Silybin A (1), silybin B (2), and isosilybin A (3), three of the seven flavonolignans that constitute silymarin, an extract of the fruits of milk thistle (Silybum marianum), were detected for the first time from a fungal endophyte, Aspergillus iizukae, isolated from the surface-sterilized leaves of S. marianum. The flavonolignans were identified using a UPLC-PDA-HRMS-MS/MS method by matching retention times, HRMS, and MS/MS data with authentic reference compounds. Attenuation of flavonolignan production was observed following successive subculturing of the original flavonolignan-producing culture, as is often the case with endophytes that produce plant-based secondary metabolites. However, production of 1 and 2 resumed when attenuated spores were harvested from cultures grown on a medium to which autoclaved leaves of S. marianum were added. The cycle of attenuation followed by resumed biosynthesis of these flavonolignans was replicated in triplicate.



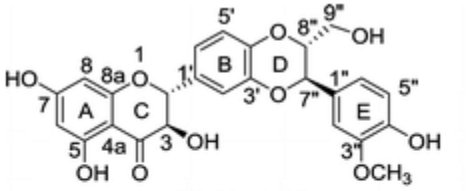
Keywords: flavonolignan | Silybum marianum | milk thistle

Article:

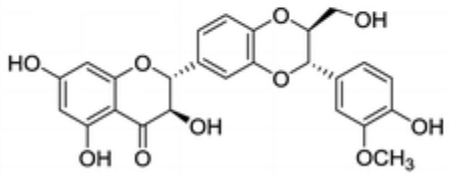
Silybum marianum (L.) Gaertn. (Asteraceae), known commonly as milk thistle, has been used for centuries for the treatment of liver disorders, including cirrhosis and chronic hepatitis.(1-7) Silymarin, the crude extract of the fruits (achenes), represents a mixture of at least seven flavonolignans [silybin A (1), silybin B (2), isosilybin A (3), isosilybin B (4), silychristin, isosilychristin, and silydianin] and the flavonoid taxifolin.(8) Silymarin has been the subject of intensive studies for its profound biological activities, particularly in the areas of cancer chemoprevention and hepatoprotection,(9-14) and our group has been studying the chemistry of flavonolignans for more than 10 years.(15-19) Recently, studies were initiated on the chemical mycology of endophytic fungi in milk thistle by examining the chemical profiles of fungal endophytes and how these are influenced by the native substrate.

Fungal endophytes are a diverse group of primarily ascomycetous fungi, which are defined functionally by their asymptomatic occurrence within plants.(20, 21) They occur in all major lineages of plants and in natural and anthropogenic communities ranging from the arctic region to the tropics.(22-24) The ecological roles and chemical interactions of endophytic fungi are currently under intense investigation, particularly in relation to their host plants.

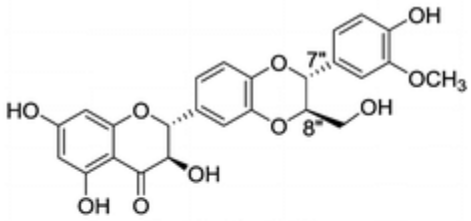
The interest in the capability of certain endophytic fungi to produce compounds associated with plant secondary metabolites has gained prominence since the report of the anticancer agent taxol from Taxomyces andreanae, a fungal endophyte that was isolated from the inner bark of Taxus brevifolia.(25) Subsequently, several research groups have explored endophytic fungi for the production of associated plant secondary metabolites, and examples include the cytotoxic agents podophyllotoxin(26, 27) and deoxypodophyllotoxin,(28) camptothecin and related analogues,(29-34) and vinblastine,(35) the herbal antidepressant hypericin and its precursor emodin,(36, 37) and the insecticides azadirachtins A and B.(38) Unfortunately, sustainable production of these and other compounds by fungal endophytes has proven difficult. Almost without exception, attenuation of production of the target compounds following subculturing of the endophytes occurs.(39) The reasons for this attenuation could be attributed to factors that stem from loss of interactions with the host plant, coexisting endophytes (of fungal and/or bacterial origin), insects, and/or herbivores,(40, 41) resulting in the silencing of genes in axenic monocultures.(42) Herein, reported for the first time is the production of two flavonolignans, silybin A (1) and silybin B (2), from an endophytic fungus that was isolated from the leaves of milk thistle. The fungus was identified as Aspergillus iizukae Sugiyama (Eurotiomycetes, Ascomycota) using molecular and morphological characterization techniques.



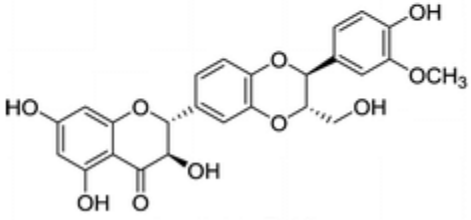
Silybin A (**1**)



Silybin B (**2**)



Isosilybin A (**3**)



Isosilybin B (**4**)

Following isolation of 21 unique fungal endophytes from leaves, stems, seeds, and roots of milk thistle, organic extracts of cultures grown on a solid medium were prepared and dereplicated against an in-house database of bioactive fungal secondary metabolites containing more than 180 compounds using a UPLC-PDA-HRMS-MS/MS method.(43) As a standard protocol, all cultures isolated in our laboratory were dereplicated before engaging in the scale-up or purification processes, so as to avoid the reisolation of known compounds, particularly mycotoxins.(43, 44) Subsequently, the 21 fungal endophytes were examined for the production of flavonolignans. To do so, a database of authentic reference standards of the major silymarin constituents was constructed using the same dereplication strategy(43) by recording HRMS, MS/MS data, UV absorption maxima, and retention times utilizing the negative-ionization mode (Table S1, Supporting Information). The MS files of the extracts, which were acquired previously for dereplicating mycotoxins, were reanalyzed for the production of flavonolignans. Among the fungal endophyte isolates, only two cultures, coded G77 and G82, had detectable ions that matched, tentatively, with four flavonolignan standards (1–4) in terms of HRMS, MS/MS fragments, and retention times. Interestingly, G77 was harvested from the leaves, while G82 was harvested from the stems; however, they both were identified later as A. iizukae. The former culture produced a higher level of signals that matched that of the flavonolignans and, as such,

was pursued further. Due to the close retention times, identical molecular ion peaks, and similar fragmentation patterns, the method(43) was unable to differentiate between either silybin A/silybin B or isosilybin A/isosilybin B. To discern between these pairs of diastereoisomers, a chromatographic method was optimized to target flavonolignans, and this was utilized when reacquiring the UPLC-PDA-HRMS-MS/MS data. Using this refined dereplication system (Table S1, Supporting Information), the compounds were identified as silybin A (1), silybin B (2), and isosilybin A (3) (Figure 1).

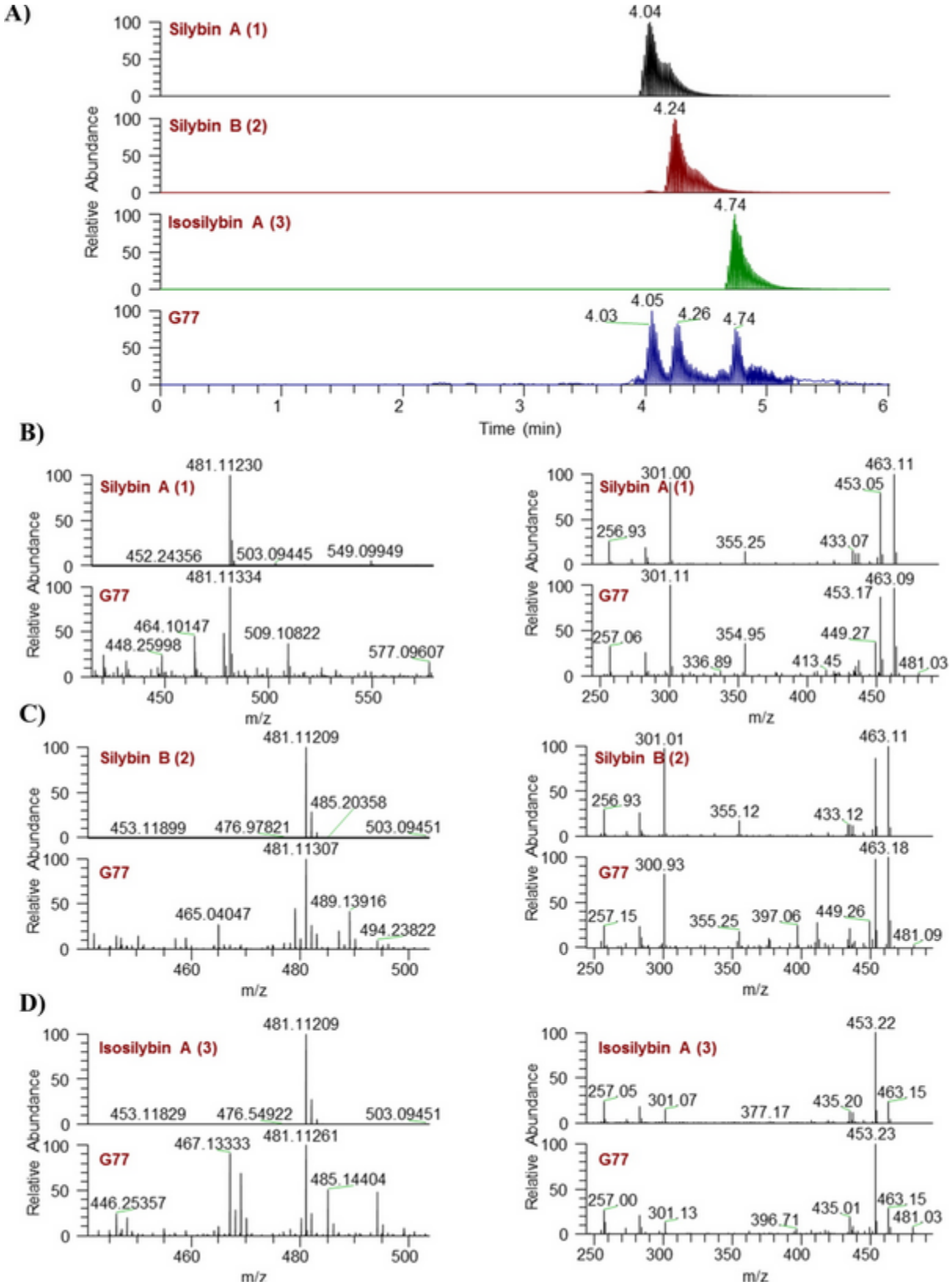

A)
Silybin A (1)
Silybin B (2)
Isosilybin A (3)
G77
4.04
4.24
4.74
4.05
4.03
4.26
4.74
Relative Abundance
Time (min)
B)
Silybin A (1)
481.11230
452.24356
503.09445
549.09949
G77
481.11334
464.10147
448.25998
509.10822
577.09607
301.00
463.11
453.05
256.93
355.25
433.07
301.11
463.09
453.17
257.06
354.95
449.27
336.89
413.45
481.03
m/z
C)
Silybin B (2)
481.11209
485.20358
453.11899
476.97821
503.09451
G77
481.11307
489.13916
465.04047
494.23822
301.01
463.11
256.93
355.12
433.12
300.93
463.18
257.15
355.25
397.06
449.26
481.09
D)
Isosilybin A (3)
481.11209
453.11829
476.54922
503.09451
G77
467.13333
481.11261
485.14404
446.25357
453.22
257.05
301.07
377.17
435.20
463.15
453.23
257.00
301.13
396.71
435.01
463.15
481.03

Figure 1. (A) (−)-ESI SIC of reference standards compared to that of an extract of the fungus A. iizukae (m/z 481); (−)-ESI high-resolution mass spectra (left) and MS/MS CID fragmentation data (right) of (B) silybin A (1), (C) silybin B (2), and (D) isosilybin A (3).

Upon successive subculturing of the original axenic flavonolignans-producing culture (A. iizukae), attenuation of the production of flavonolignans was observed. In an attempt to stimulate the fungus to biosynthesize these compounds, an experiment was designed by growing A. iizukae on potato dextrose agar (Difco) to which autoclaved milk thistle leaves were added (Figure 2). Following growth for one month, spores (asexual conidia) were transferred aseptically from the plate and were used either to inoculate the rice medium directly or to inoculate a liquid culture containing 2% soy peptone, 2% dextrose, and 1% yeast extract (YESD medium). After seven days, this liquid culture was used to inoculate the rice medium. In addition, an agar plug from a culture of A. iizukae that was grown on potato dextrose agar (PDA) medium without autoclaved milk thistle leaves was used to inoculate the rice medium directly. All three cultures were grown on rice for 21 days and then extracted and analyzed by UPLC-PDA-HRMS using the system optimized for flavonolignans. The flavonolignans silybin A (1) and silybin B (2) were detected only in the solid medium that was inoculated directly by spores from A. iizukae grown on medium that included autoclaved milk thistle leaves. Upon subculturing of this flavonolignan-producing culture, the production of flavonolignans was attenuated again. However, flavonolignan production was restimulated by growing the attenuated culture on PDA medium with autoclaved milk thistle leaves and harvesting spores for inoculation on rice medium (Figure 3). Importantly, the conidiophores were up to 1.5 mm long (Figure S1, Supporting Information).(45) Hence, it was easy to harvest a small amount of spores using the tip of a sterile bent sewing needle, which was attached to a 20 cm long needle holder, without touching the surface of the agar. Moreover, the flavonolignans were not detected in organic extracts of the milk thistle leaves used in this study.

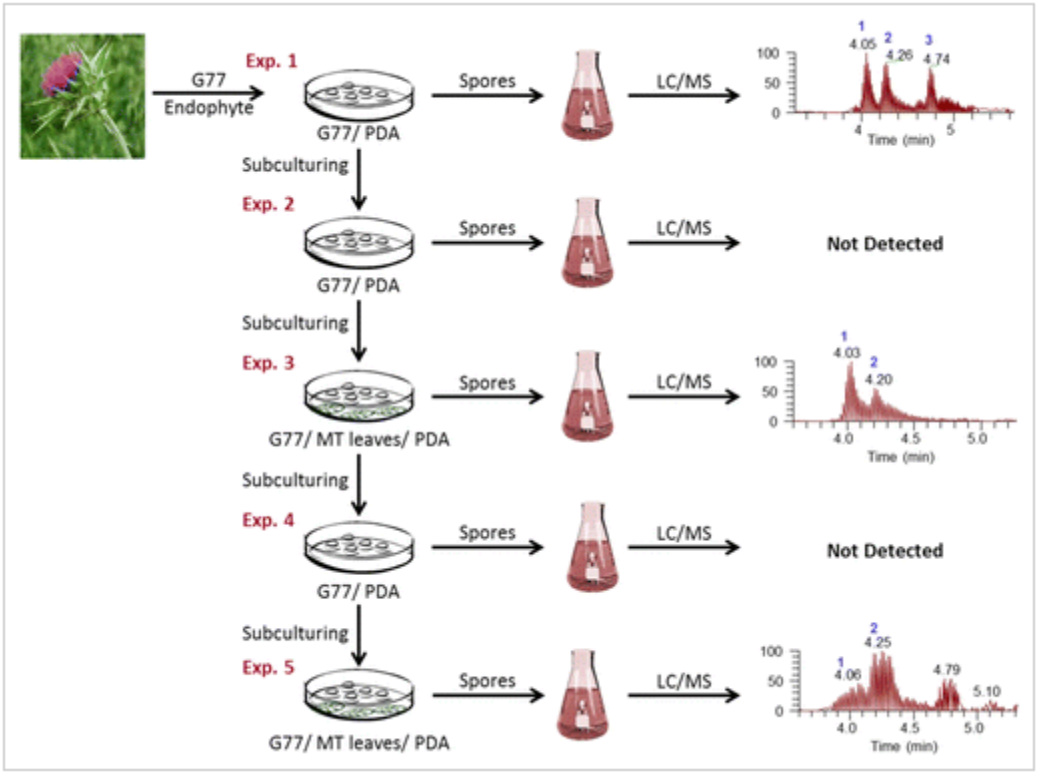


Figure 3. Flavonolignan production by Aspergillus iizukae upon subculturing. Abbreviations: G77, A. iizukae; MT, milk thistle; PDA, potato dextrose agar; 1, silybin A; 2, silybin B; and 3, isosilybin A.

Obvious questions could be raised as to where the flavonolignans originated. The leaves that were amended to the cultures were ruled out for three reasons: there were no leaves in the medium when the flavonolignans were observed originally, compounds 1–4 were not detected in the leaves, and, most importantly, the spores were removed from the Petri dishes without touching the agar medium before being transferred to the rice medium for growth. Also, contamination of both glassware and instrumentation was dismissed for at least three reasons: solvent blanks were injected between runs on the UPLC-PDA-HRMS-MS/MS system, the column on the UPLC was used exclusively for fungal samples, and the experiments between amended cultures (i.e., experiments 2 and 4 in Figure 3) did not show the production of flavonolignans. Further to this point, when the flavonolignans were observed originally, via a random screening of more than a score of fungal extracts, they were observed in only two samples. Later, it was determined that these isolates were identical via morphological and molecular methods. Finally, a misidentification of the flavonolignans was excluded, as the reference standards were characterized thoroughly.(15, 17, 47) Having eliminated all of those possibilities, we concluded that the leaves in the medium stimulated the biosynthesis of flavonolignans, such that these compounds could be observed upon growth of the fungus from spores on solid medium.

Discovery of endophytic fungi that can produce the same compounds of their associated host plants could be important from chemical and evolutionary perspectives. These may serve as a sustainable and alternative source for biologically active secondary metabolites that are produced

by endangered or difficult-to-collect plant species. This has been attempted with a variety of metabolites.(25, 27, 37) To date, the relative production levels in endophytic cultures have not been large enough to be considered viable. Future studies that explore adding substrates to culture media may improve this. From an evolutionary viewpoint, a horizontal transfer of genes encoding for secondary metabolites could have occurred between the host plant and its associated endophytic fungi.(42) If so, then understanding how plant defenses against herbivores and pathogens evolved may need to also consider the impact of endophytic symbiants. Genomic studies of secondary metabolite gene clusters may provide additional information on how endophytic fungi are capable of producing plant-associated bioactive compounds and if there are cryptic gene clusters in fungal endophytes that are capable of yielding plant metabolic products. Such studies could yield fungal cultures that are amenable to genetic manipulation for the production of non-natural analogues of plant-based secondary metabolites.

Experimental Section

General Experimental Procedures

HRESIMS was performed on a Thermo LTQ Orbitrap XL mass spectrometer (ThermoFisher, San Jose, CA, USA) equipped with an electrospray ionization source in the negative ionization mode. Source conditions in the negative ionization mode were 275 °C for capillary temperature, 3.5 kV for source voltage, 42 V for capillary voltage, and 110 V for tube lens. Nitrogen was utilized for the sheath and auxiliary gases and set to 20 and 10 arb, respectively. Two scan events were carried out: full scan (100–2000) and ion trap MS/MS of the most intense ion from the parent mass list utilizing CID with a normalized collision energy of 30. Thermo Scientific Xcalibur 2.1 software was used for instrument control and data analysis. UPLC was carried out on a Waters Acquity system [using an HSS T3 (2.1 × 100 mm, 1.8 μm) column (Waters Corp., Milford, MA, USA) equilibrated at 50 °C]. A mobile phase consisting of MeOH–H2O (acidified with 0.1% formic acid) was used, starting with 30:70 then increasing linearly to 55% MeOH within 5 min, then returning to the starting conditions within 0.1 min, and holding for 0.9 min, for a total run time of 6 min and a flow rate of 0.6 mL/min. An Acquity UPLC photodiode array detector was used to acquire PDA spectra, which were collected from 201 to 499 nm with 3.6 nm resolution. Quantification of flavonolignans was performed using the same UPLC-MS method based on a linear calibration curve (r2 = 1.00) of five concentrations (5, 10, 20, 40, and 80 μg/mL) of an authentic standard of silybin B (2) (Figure S1, Supporting Information).(17)

Isolation and Fermentation of the Fungal Endophyte

A healthy, asymptomatic plant of Silybum marianum (milk thistle) was obtained from Horizon Herbs (lot #6510), a private farming company located in Williams, OR, USA, in August 2011. A voucher specimen of the plant material was deposited in the Herbarium of the University of North Carolina at Chapel Hill (NUC602014). The stems, leaves, roots, and seeds of the plant were cut into small pieces (approximately 2–5 mm in length) and washed in tap water. Subsequently, the segments were surface-sterilized by sequential immersion in 95% EtOH for 10 s, NaClO (10–15% available chlorine, Sigma) for 2 min, and 70% EtOH for 2 min. The plant segments were transferred under aseptic conditions onto 2% malt extract agar (MEA, Difco; 20 g MEA, 1000 mL sterile distilled water with the antibiotics streptomycin sulfate 250 mg/L and

penicillin G 250 mg/L). To test the efficacy of the surface-sterilization procedure and to confirm that emergent fungi were endophytic and not of epiphytic origin, individual surface-sterilized leaf, stem, root, and seed segments were spread and then removed on separate MEA plates with antibiotics; the absence of fungal growth on the nutrient medium confirmed the effectiveness of the sterilization procedure.(48) Plates were sealed with Parafilm and incubated at room temperature until emergent fungal colonies were observed.

One of the endophytes from milk thistle leaves was accessioned as G77. The cultures of G77 were subsequently grown on 2% MEA, PDA (Difco), and YESD media. After 14–21 days, spores of the fungus were used to inoculate 50 mL of a rice medium, prepared using 25 g of rice and twice the volume of rice with H2O in a 250 mL Erlenmeyer flask. This inoculated medium was incubated at 22 °C until the culture showed good growth (approximately 14 days). A voucher culture of the G77 strain is maintained in the Department of Chemistry and Biochemistry culture collection at the University of North Carolina at Greensboro.

Identification of the Fungal Strain

For molecular identification, DNA was extracted from fresh cultures of G77 grown on PDA amended with antibiotics (streptomycin sulfate 250 mg/L, penicillin G 250 mg/L, distilled water 1 L; antibiotics were added to the molten agar immediately after autoclaving). The nuclear internal transcribed spacers and intervening 5.8S gene, which together have been proposed as a barcoding marker for fungi,(49) along with the D1/D2 regions of the adjacent nuclear ribosomal large subunit,(50) were amplified and sequenced following protocols published previously.(51) The consensus sequence of the ITS region was submitted for a BLAST search using the NCBI GenBank database to obtain species-level information. The ITS sequence was then utilized in combination with the D1/D2 regions of the LSU for subsequent phylogenetic analysis. The top BLAST matches for G77 indicated similarities to sequences of A. iizukae (EF669597; identities = 558/562 (99%), gaps = 3/562 (0%)) and A. iizukae (EF669596; identities = 558/562 (99%), gaps = 3/562 (0%)). GenBank sequence EF669597 belongs to NRRL 3750, which is the type isolate of A. iizukae.(52) The ITS sequence of G77 was also searched against the Fungal Barcoding database (http://www.fungalbarcoding.org/) using the pairwise sequence alignment. Results also suggested a 99% sequence similarity with an isolate of A. iizukae (DTO 065-G6). After the initial BLAST searches, top BLAST hits from the GenBank were downloaded and incorporated into a multiple sequence alignment of combined ITS and D1/D2 regions of LSU data using MUSCLE(53) with default parameters in operation. MUSCLE was implemented using the program Seaview v. 4.1.(54) Maximum likelihood (ML) analyses were then performed using a RAxML v. 7.0.4(55, 56) run on the CIPRES Portal v. 2.0(57) with the default rapid hill-climbing algorithm and GTR model employing 1000 fast bootstrap searches. In addition, Bayesian analysis was also performed to assess support for tree topology using MrBayes.(58) The strain G77 was nested in a cluster with sequences of A. iizukae including the type isolate (NRRL 3750; EF669597) with moderate bootstrap support within the section Flavipedes (Figure S3, Supporting Information). On the basis of the results of the BLAST search and ML analysis, strain G77 was identified as A. iizukae. The combined ITS and partial LSU sequence for A. iizukae was deposited in GenBank (accession No. AB859956). A. iizukae Sugiyama was reported originally in soil from stratigraphic drilling core, Gymna Prefecture, Fujioka, Japan.(45) The endophytic strain isolated from milk thistle leaves was morphologically identical to the

protologue.(45) The same analysis was conducted on G82, an isolate that was harvested from the stems of milk thistle; the taxonomy and phylogeny of G77 and G82 were identical.

Extraction

To each solid fermentation culture of A. iizukae, 60 mL of 1:1 MeOH–CHCl3 was added. The culture was chopped with a spatula and shaken overnight (∼16 h) at ∼100 rpm at room temperature. The sample was filtered under vacuum, and the remaining residues were washed with 10 mL of 1:1 MeOH–CHCl3. To the filtrate were added 90 mL of CHCl3 and 150 mL of H2O; the mixture was stirred for 30 min and then transferred into a separatory funnel. The bottom layer was drawn off and evaporated to dryness. The dried organic extract was dissolved in 100 mL of 1:1 MeOH–CH3CN and 100 mL of hexanes. The biphasic solution was transferred to a separatory funnel and shaken vigorously. The MeOH–CH3CN layer was drawn off and evaporated to dryness under vacuum to yield 47.3, 301.7, and 575.9 mg of extract for the first, third, and fifth cultures, respectively. For UPLC-PDA-HRMS-MS/MS analysis, a submilligram aliquot of each extract was dissolved in equal volumes of MeOH and dioxane to obtain a final concentration of 2 mg/mL in a total volume of 150 μL. To ensure that flavonolignans were not carried over between successive injections, consecutive blanks of solvents were injected between samples during analysis on the UPLC-PDA-HRMS-MS/MS system.

Using the same protocols and solvent systems that were used for the fungal cultures, approximately 0.5 g of dry, finely ground milk thistle leaves was extracted and fractionated; this was repeated on three separate samples of leaves. An aliquot of each of the dried MeOH–CH3CN extracts was analyzed for flavonolignans using the UPLC-PDA-HRMS-MS/MS method.

Supporting Information

Chemical formulas, retention times, UV absorption maxima, (−)-ESI HRMS, and (−)-ESI CID MS/MS of silymarin constituents, stereomicrographs of A. iizukae culture, external calibration curve for silybin B (2), and molecular phylogenetic tree of A. iizukae. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

T. El-Elimat and H. A. Raja contributed equally to this work.

The authors declare no competing financial interest.

Acknowledgment

The authors thank D. Hayes of UNCG and R. Cech of Horizon Herbs for assistance with the plant material acquisition. This research was supported by a Biotechnology Research Grant (2011-BRG-1206) from the North Carolina Biotechnology Center. The high-resolution mass spectrometry data were acquired in the Triad Mass Spectrometry Laboratory at the University of North Carolina at Greensboro. Sequence data were generated at the mycology laboratory of Dr. A. N. Miller, Illinois Natural History Survey, University of Illinois at Urbana–Champaign.

References

1. Polyak, S. J.; Oberlies, N. H.; Pecheur, E. I.; Dahari, H.; Ferenci, P.; Pawlotsky, J. M. Antiviral Ther. 2013, 18, 141– 147
2. Abenavoli, L.; Capasso, R.; Milic, N.; Capasso, F. Phytother. Res. 2010, 24, 1423– 1432
3. Deep, G.; Agarwal, R. Cancer Metastasis Rev. 2010, 29, 447– 463
4. Ramasamy, K.; Agarwal, R. Cancer Lett. 2008, 269, 352– 362
5. Agarwal, R.; Agarwal, C.; Ichikawa, H.; Singh, R. P.; Aggarwal, B. B. Anticancer Res. 2006, 26, 4457– 4498
6. Comelli, M. C.; Mengs, U.; Schneider, C.; Prosdocimi, M. Integr. Cancer Ther. 2007, 6, 120– 129
7. Gazak, R.; Walterova, D.; Kren, V. Curr. Med. Chem. 2007, 14, 315– 338
8. Kroll, D. J.; Shaw, H. S.; Oberlies, N. H. Integr. Cancer Ther. 2007, 6, 110– 119
9. Davis-Searles, P. R.; Nakanishi, Y.; Kim, N. C.; Graf, T. N.; Oberlies, N. H.; Wani, M. C.; Wall, M. E.; Agarwal, R.; Kroll, D. J. Cancer Res. 2005, 65, 4448– 4457
10. Deep, G.; Oberlies, N. H.; Kroll, D. J.; Agarwal, R. Carcinogenesis 2007, 28, 1533– 1542
11. Deep, G.; Gangar, S. C.; Oberlies, N. H.; Kroll, D. J.; Agarwal, R. Mol. Carcinog. 2010, 49, 902– 912
12. Deep, G.; Gangar, S. C.; Rajamanickam, S.; Raina, K.; Gu, M.; Agarwal, C.; Oberlies, N. H.; Agarwal, R. Plos One 2012, 7.
13. Polyak, S. J.; Morishima, C.; Lohmann, V.; Pal, S.; Lee, D. Y. W.; Liu, Y. Z.; Graf, T. N.; Oberlies, N. H. Proc. Natl. Acad. Sci. U.S.A. 2010, 107, 5995– 5999
14. Wagoner, J.; Morishima, C.; Graf, T. N.; Oberlies, N. H.; Teissier, E.; Pecheur, E. I.; Tavis, J. E.; Polyak, S. J. Plos One 2011, 6.
15. Napolitano, J. G.; Lankin, D. C.; Graf, T. N.; Friesen, J. B.; Chen, S.-N.; McAlpine, J. B.; Oberlies, N. H.; Pauli, G. F. J. Org. Chem. 2013, 78, 2827– 2839
16. Sy-Cordero, A. A.; Graf, T. N.; Runyon, S. P.; Wani, M. C.; Kroll, D. J.; Agarwal, R.; Brantley, S. J.; Paine, M. F.; Polyak, S. J.; Oberlies, N. H. Bioorg. Med. Chem. 2013, 21, 742– 747
17. Graf, T. N.; Wani, M. C.; Agarwal, R.; Kroll, D.; Oberlies, N. H. Planta Med. 2007, 73, 1495– 1501
18. Althagafy, H. S.; Meza-Avina, M. E.; Oberlies, N. H.; Croatt, M. P. J. Org. Chem. 2013, 78, 7594– 7600
19. Althagafy, H. S.; Graf, T. N.; Sy-Cordero, A. A.; Gufford, B. T.; Paine, M. F.; Wagoner, J.; Polyak, S. J.; Croatt, M. P.; Oberlies, N. H. Bioorg. Med. Chem. 2013, 21, 3919– 3926
20. Carroll, G. Ecology 1988, 69, 2– 9
21. Stone, J. K.; Polishook, J. D.; White, J. F., Jr. In Biodiversity of Fungi: Inventory and Monitoring Methods; Mueller, G. M.; Bills, G. F.; Foster, M. S., Eds.; Elsevier Academic Press: Burlington, MA, USA, 2004; pp 241– 270.
22. Petrini, O. In Microbial Ecology of Leaves; Andrews, J. H.; Hirano, S. S., Eds.; Springer-Verlag: New York, 1991; pp 179– 197.
23. Stone, J. K.; Bacon, C. W.; White, J. F., Jr. In Microbial Endophytes; Bacon, C. W.; White, J. F., Jr., Eds.; Marcel Dekker: New York, 2000; pp 3– 29.
24. Arnold, A. E. Fungal Biol. Rev. 2007, 21, 51– 66
25. Stierle, A.; Strobel, G.; Stierle, D. Science 1993, 260, 214– 216
26. Eyberger, A. L.; Dondapati, R.; Porter, J. R. J. Nat. Prod. 2006, 69, 1121– 1124

27. Puri, S. C.; Nazir, A.; Chawla, R.; Arora, R.; Riyaz-ul-Hasan, S.; Amna, T.; Ahmed, B.; Verma, V.; Singh, S.; Sagar, R.; Sharma, A.; Kumar, R.; Sharma, R. K.; Qazi, G. N. J. Biotechnol. 2006, 122, 494– 510
28. Kusari, S.; Lamshoft, M.; Spiteller, M. J. Appl. Microbiol. 2009, 107, 1019– 1030
29. Shweta, S.; Gurumurthy, B. R.; Ravikanth, G.; Ramanan, U. S.; Shivanna, M. B. Phytomedicine 2013, 20, 337– 342
30. Puri, S. C.; Verma, V.; Amna, T.; Qazi, G. N.; Spiteller, M. J. Nat. Prod. 2005, 68, 1717– 1719
31. 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
32. Rehman, S.; Shawl, A. S.; Kour, A.; Sultan, P.; Ahmad, K.; Khajuria, R.; Qazi, G. N. Nat. Prod. Res. 2009, 23, 1050– 1057
33. Shweta, S.; Zuehlke, S.; Ramesha, B. T.; Priti, V.; Kumar, P. M.; Ravikanth, G.; Spiteller, M.; Vasudeva, R.; Shaanker, R. U. Phytochemistry 2010, 71, 117– 122
34. Kusari, S.; Zuhlke, S.; Spiteller, M. J. Nat. Prod. 2009, 72, 2– 7
35. Guo, B.; Li, H.; Zhang, L. J. Yunnan Univ. 1998, 20, 214– 215
36. Kusari, S.; Zuhlke, S.; Kosuth, J.; Cellarova, E.; Spiteller, M. J. Nat. Prod. 2009, 72, 1825– 1835
37. Kusari, S.; Lamshoft, M.; Zuhlke, S.; Spiteller, M. J. Nat. Prod. 2008, 71, 159– 162
38. Kusari, S.; Verma, V. C.; Lamshoeft, M.; Spiteller, M. World J. Microbiol. Biotechnol. 2012, 28, 1287– 1294
39. Gurudatt, P. S.; Priti, V.; Shweta, S.; Ramesha, B. T.; Ravikanth, G.; Vasudeva, R.; Amna, T.; Deepika, S.; Ganeshaiah, K. N.; Shaanker, R. U.; Puri, S.; Qazi, N. Curr. Sci. 2010, 98, 1006– 1010
40. Kusari, S.; Hertweck, C.; Spiteller, M. Chem. Biol. 2012, 19, 792– 798
41. Kusari, S.; Pandey, S. P.; Spiteller, M. Phytochemistry 2013, 91, 81– 87
42. Sachin, N.; Manjunatha, B. L.; Kumara, P. M.; Ravikanth, G.; Shweta, S.; Suryanarayanan, T. S.; Ganeshaiah, K. N.; Shaanker, R. U. Curr. Sci. 2013, 104, 178– 182
43. El-Elimat, T.; Figueroa, M.; Ehrmann, B. M.; Cech, N. B.; Pearce, C. J.; Oberlies, N. H. J. Nat. Prod. 2013, 76, 1709– 1716
44. Sy-Cordero, A. A.; Graf, T. N.; Wani, M. C.; Kroll, D. J.; Pearce, C. J.; Oberlies, N. H. J. Antibiot. 2010, 63, 539– 544
45. Sugiyama, J. J. Fac. Sci., Univ. Tokyo, Sect. 3 1967, 9 (Parts 10 and 11) 374– 405
46. Kusari, S.; Zuhlke, S.; Spiteller, M. J. Nat. Prod. 2011, 74, 764– 775
47. Sy-Cordero, A. A.; Day, C. S.; Oberlies, N. H. J. Nat. Prod. 2012, 75, 1879– 1881
48. Schulz, B.; Wanke, U.; Draeger, S.; Aust, H. J. Mycol. Res. 1993, 97, 1447– 1450
49. Schoch, C. L.; Seifert, K. A.; Huhndorf, S.; Robert, V.; Spouge, J. L.; Levesque, C. A.; Chen, W.Fungal Barcoding Consortium Proc. Natl. Acad. Sci. U.S.A. 2012, 109, 6241– 6246
50. Guadet, J.; Julien, J.; Lafay, J. F.; Brygoo, Y. Mol. Biol. Evol. 1989, 6, 227– 242
51. El-Elimat, T.; Figueroa, M.; Raja, H. A.; Graf, T. N.; Adcock, A. F.; Kroll, D. J.; Day, C. S.; Wani, M. C.; Pearce, C. J.; Oberlies, N. H. J. Nat. Prod. 2013, 76, 382– 387
52. Peterson, S. W. Mycologia 2008, 100, 205– 226
53. Edgar, R. C. Nucleic Acids Res. 2004, 32, 1792– 1797
54. Gouy, M.; Guindon, S.; Gascuel, O. Mol. Biol. Evol. 2010, 27, 221– 224

55. Stamatakis, A.; Aberer, A. J.; Goll, C.; Smith, S. A.; Berger, S. A.; Izquierdo-Carrasco, F. Bioinformatics 2012, 28, 2064– 2066
56. Stamatakis, A. Bioinformatics 2006, 22, 2688– 2690
57. Miller, M. A.; Pfeiffer, W.; Schwartz, T. In Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, LA, November 14, 2010; pp 1– 8.
58. Huelsenbeck, J. P.; Ronquist, F. In Statistical Methods in Molecular Evolution; Nielsen, R., Ed.; Springer: New York, NY, USA, 2005; pp 183– 232.