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Article

Insights into the Chemical Diversity of Selected Fungi from the Tza Itzá Cenote of the Yucatan Peninsula

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ABSTRACT: Cenotes are habitats with unique physical, chemical, and biological features. Unexplored microorganisms from these sinkholes represent a potential source of bioactive molecules. Thus, a series of cultivable fungi (*Aspergillus* spp. NCA257, NCA264, and NCA276, *Stachybotrys* sp. NCA252, and *Cladosporium* sp. NCA273) isolated from the cenote Tza Itzá were subjected to chemical, coculture, and metabolomic analyses. Nineteen compounds were obtained and tested for their antimicrobial potential against ESKAPE pathogens, *Mycobacterium tuberculosis*, and nontuberculous mycobacteria. In particular, phenylspirodrimanes from *Stachybotrys* sp. NCA252 showed significant activity against MRSA, MSSA, and mycobacterial strains. On the other hand, the absolute configuration of the new compound 17-deoxy-aspergillin PZ (**1**) isolated from *Aspergillus* sp. NCA276 was established via single-crystal X-ray crystallography. Also, the chemical analysis of the cocultures between *Aspergillus* and *Cladosporium* strains revealed the production of metabolites that were not present or were barely detected in the monocultures. Finally, molecular networking analysis of the LC-MS-MS/MS data for each fungus was used as a tool for the annotation of additional compounds, increasing the chemical knowledge on the corresponding fungal strains. Overall, this is the first systematic chemical study on fungi isolated from a sinkhole in Mexico.

INTRODUCTION

One of the most famous geological features in the Yucatan peninsula is the ring of cenotes (from the Maya word *ts'onot*), which is a group of sinkholes formed by the process of karstification.[1] The origin of cenotes is related to the impact of the Chicxulub asteroid around 66 millions of years ago, which caused changes in the karst features.[2,3] These habitats are physically and chemically unique due to the phototrophic activity of its microbial communities. In particular, the carbon and sulfur cycles completed by different microbes affect the sulfate reduction into aqueous sulfide, accelerating the limestone dissolution, and increasing alkalinity.[4,5] In addition, most of the cenotes have hydraulic connections or networks across the region, and the microbial communities, especially freshwater fungi and bacteria, play a key role in keeping the balance of the entire ecosystem.[6]

Metagenomic analysis of soil samples from freshwater environments in the Yucatan peninsula revealed a remarkable microbial diversity, where fungi (ascomycetes) prevail over bacteria (actinobacteria) in terms of biomass production and enzymatic substrate degradation.[7] Furthermore, the biosynthetic potential of these communities of microbes was assessed using bioinformatics tools coupled with direct amplification of environmental DNA.[8−10] The number of studies demonstrating the potential of microorganisms from cenotes for the discovery of bioactive natural products is scarce. Such studies have focused on the biological activity of extracts, exhibiting a high percentage of hits (~81%). About half of the extracts showed varied activities, including insecticidal and nematoxic.[11−13] To date, the only formal chemical study reported from a fungal strain isolated from plant litter submerged in a sinkhole in Merida, Yucatan, led to the discovery of the novel hexahydroacremonintriol. This compound showed moderate insecticidal activity against phytophagous *Myzus persicae*, and *Rhopalosiphum padi*.[14]

The number of microorganisms from this region seems to be underexplored. The Yucatan peninsula thus remains an invaluable source of biological diversity for bioprospecting purposes.[15,16] In this study, the chemical diversity and antimicrobial properties of a series of cultivable fungi isolated

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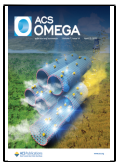

Figure 1. Location of cenote Tza Itzá (20°43′50.27″ N, 89°27′56.82″ W) in the Yucatan peninsula.

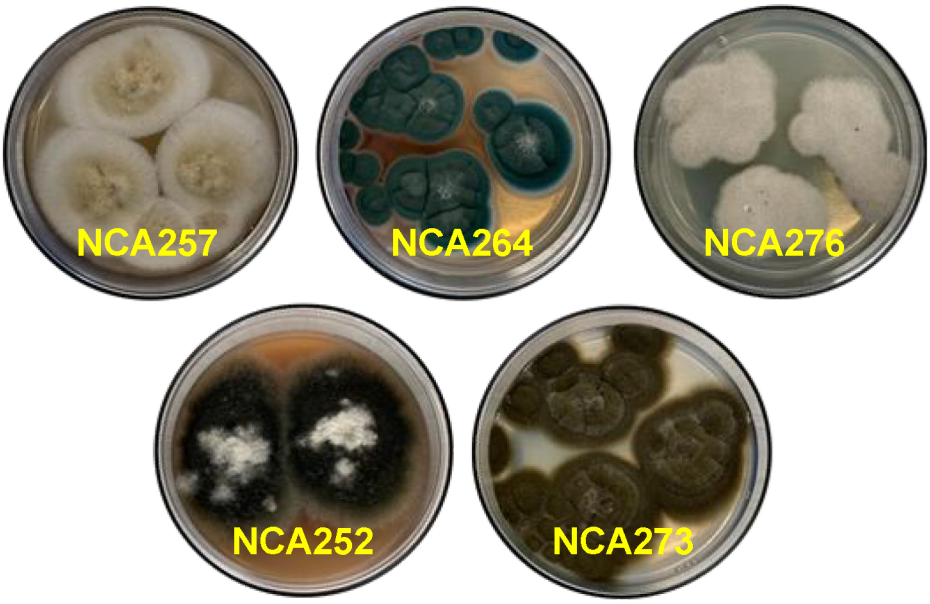

Figure 2. Fungal isolates from cenote Tza Itzá on PDA medium: *Aspergillus* spp. NCA257, NCA264, and NCA276; *Stachybotrys* sp. NCA252; and *Cladosporium* sp. NCA273.

from the Tza Itzá cenote were explored by combining conventional chemical studies, cocultures analysis, and metabolomics (Figure 1). The isolation of several compounds from single-strain cultures demonstrated their potential to produce interesting chemistry. In addition, the analysis of cocultures led to the identification of compounds that were not present, or were barely detected, in the monocultures. Moreover, molecular networking (MN) analysis of the liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) data of each fungus, allowed the annotation of additional compounds beyond to the isolated. Finally, several isolated compounds showed significant antibacterial activity against ESKAPE pathogens, *Mycobacterium tuberculosis*, and nontuberculous mycobacterias.

RESULTS AND DISCUSSION

Fungal Strains Isolation and Identification. Five culturable fungal strains were isolated from sediments samples collected in the cenote Tza Itzá. The taxonomic identity of the strains was determined by molecular sequencing of the ITS rDNA, followed by BLAST search and maximum likelihood analysis.[17,18] The strains were identified as *Aspergillus*

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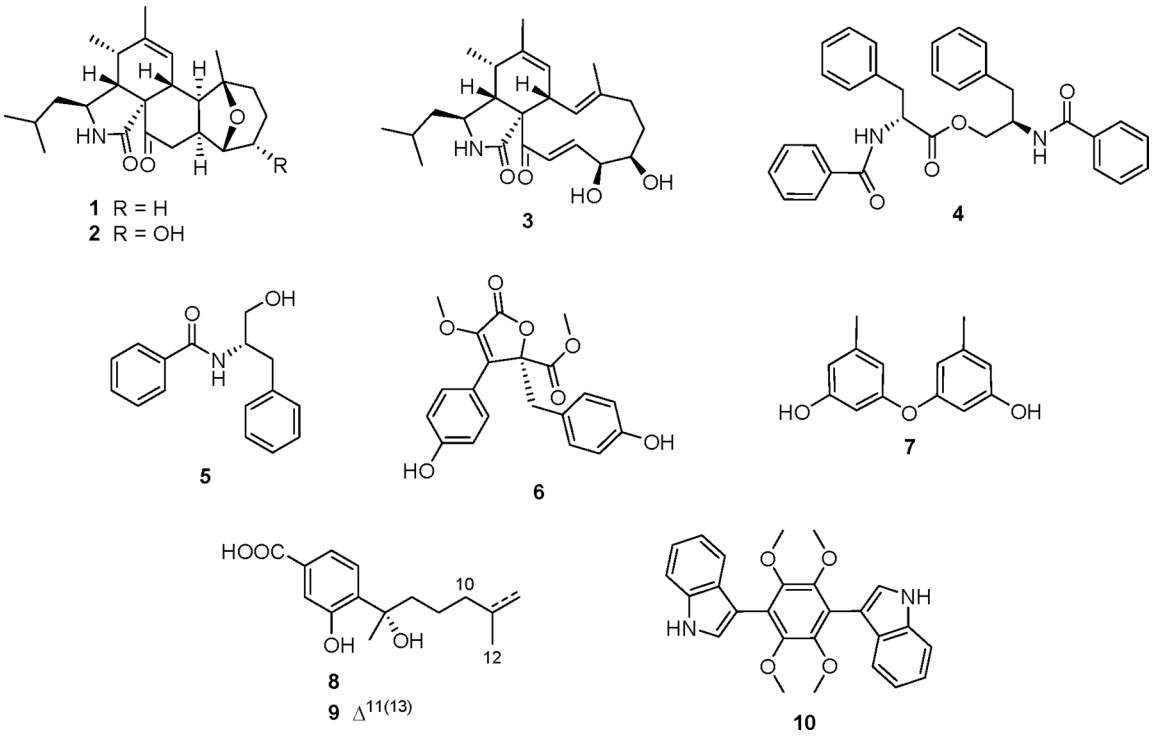

Figure 3. Isolated compounds from *Aspergillus* spp. NCA257, NCA264, and NCA276.

(NCA257, NCA264, and NCA276), *Stachybotrys* (NCA252), and *Cladosporium* (NCA273) (Figure 2 and Table S1, Supporting Information). Next, and as part of a program to explore the chemical diversity and antimicrobial potential of fungal species isolated from unexplored areas of Mexico, isolated strains were subjected to chemical, biological, and metabolomic analyses.

Chemical Study and Molecular Networking Analysis of *Aspergillus* spp. The chemical study of the defatted extracts from moist rice cultures of the *Aspergillus* spp. yielded a new compound, 17-deoxy-aspergillin PZ (**1**),[19] and the known aspergillin PZ (**2**),[20] aspochalasin D (**3**),[21] asperphenamate (**4**),[22] *N*-benzoyl-L-phenylalaninol (**5**),[23] and 2-*O*-methylbutyrolactone II (**6**)[24] from strain NCA276; diorcinol (**7**),[25] sydonic acid (**8**),[26] and 11-dehydrosydonic acid (**9**)[27] from NCA264; and asterriquinol D dimethyl ether (**10**)[28] from NCA257 (Figure 3). All known compounds were elucidated by comparison with reported spectroscopic data (Supporting Information). Compound **1** was originally isolated from a jar fermentation of a presumably *Aspergillus* species, and its planar structure was established by NMR analysis.[19] However, its absolute configuration had not been determined before. Suitable colorless crystals (Figure S1, Supporting Information) of **1** were obtained for X-ray structural determination and its absolute configuration was established as 3*S*,3a*R*,4*S*,6a-*S*,8a*S*,9*R*,13*S*,13a*R*,13b*R*-**1** (Figure 4).

Next, the organic extracts of these strains were analyzed by ultrahigh-performance liquid chromatography tandem high-resolution electrospray mass spectrometry (UPLC-HRESIMS-MS/MS). Then, metabolomic analyses were performed using an in-house dereplication procedure[29,30] and the Global Natural Products Social (GNPS) platform to perform feature-based molecular networking (FBMN) and spectral library search.[31−33] The strain NCA276 showed the largest and most chemically diverse MN. Its metabolite features were

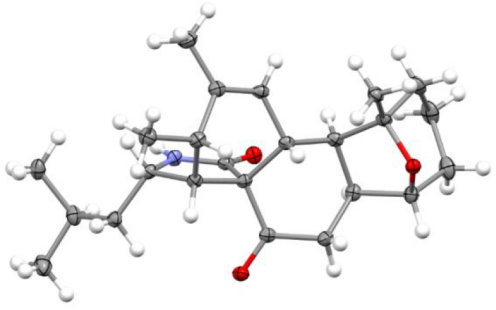

Figure 4. Displacement ellipsoid plot (50% probability level) of **1** at 100(2) K.

grouped into 800 nodes arranged in 22 clusters or chemical families with >3 nodes per cluster, 29 with two nodes, and 506 singletons (Figure 5). Chemical ontology analysis by the MolNetEnhancer GNPS tool classified the molecular features in six classes of compounds (Figure 5). Detailed analysis of the aspochalasins (cytochalasans) cluster allowed to manually annotate the isolated compounds **1**−**3** using their HRMS-MS/MS data along with a series of related compounds (aspochalasins I and K, and flavichalasin N)[34,35] annotated by GNPS (Figure 5 and Table 1). In the phenylalanine and derivatives cluster, **4** and its precursor **5** were annotated (Figure 5). Lastly, emeriphenolicin F[36] and erythroskyrin[37] were found by GNPS in the glutamic and derivatives and furofuranes nodes (Figure 5). For strain NCA264, FBMN analysis grouped the molecular features (591 nodes) into 9 classes of compounds clustered in 29 chemical families with >3 nodes per cluster, 29 with two nodes, and 542 singletons (Figure 6). The biggest family in the network belongs to the sesquiterpenoids, where **8**, **9**, and its derivative (*S*)-10-hydroxysydonic acid[38] were annotated (Figure 6). In addition, **7** was successfully annotated by GNPS in the phenyl-

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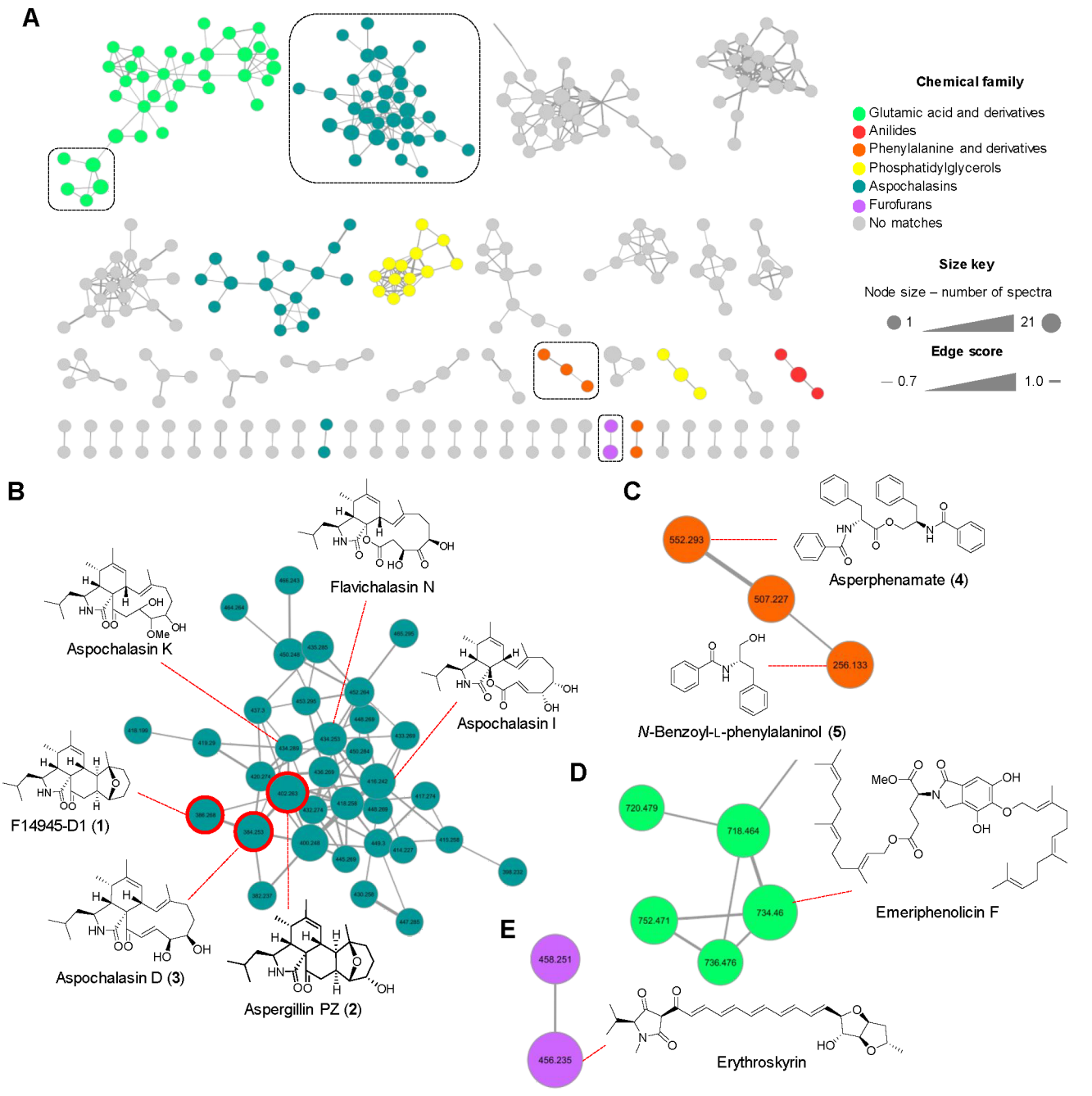

Figure 5. Metabolomic analysis of *Aspergillus* sp. NCA276. (A) FBMN (>2 nodes per cluster) and (B–E) selected clusters with nodes showing the compounds manually annotated (red circles) and by GNPS library search. Singletons (no molecular relatives) are not shown in the network.

propanoids and polyketides family (Table 1). Finally, MN of strain NCA257 showed 154 nodes grouped in 11 clusters with >3 nodes per cluster, 3 with two nodes, and 99 singletons. Interestingly, FBMN analysis did not show any structural families, but the annotation of **10** was possible using its HRMS-MS/MS data (Figure 7 and Table 1).

Chemical Study and Molecular Networking Analysis of *Stachybotrys* sp. NCA252 and *Cladosporium* sp. NCA273. From the organic extract of *Stachybotrys* sp. NCA252 culture, the isocoumarin, *O*-methylmellein (**11**),[39] the dolabellane-type diterpenoids, atranones A and B (**12** and **13**),[40] and the phenylspirodrimanes, stachybotrolide acetate (**14**), stachybotrydial acetate (**15**), and stachybotrolide (**16**),[41,42] were isolated (Figure 8 and Supporting Information). Compounds **12** and **13** are commonly observed in the chemotype A of *Stachybotrys* spp., and no compounds from chemotype S (e.g., macrocyclic trichothecenes such as satratoxins and roridins) were detected in the NCA252 strain.[43,44] In addition, the interconversion of **15** into lactone **14** was observed by NMR[45] (Figure S44). Finally, this is the first report of the isolation of **11** in a fungus of the *Stachybotrys* genera.

GNPS analysis of this strain grouped the metabolite features into 325 nodes arranged in 12 clusters with >3 nodes per cluster, 17 with two nodes, and 103 singletons. Chemical ontology analysis showed the presence of seven classes of compounds (Figure 9). Detailed analysis of the benzofurans and phtalides nodes allowed the manual annotation of **14** and

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Table 1. Chemical Annotation of Metabolites from *Aspergillus* spp

compound	observed ion (m/z)[d]	adduct	molecular formula	exact mass (m/z)[e]	mass accuracy (ppm)
Aspergillus sp. NCA276					
17-deoxy-aspergillin PZ (**1**)[a,b]	386.268	$[M+H]^+$	$C_{24}H_{36}NO_3$	386.2682	−2.0
aspergillin PZ (**2**)[a,b]	402.263	$[M+H]^+$	$C_{24}H_{36}NO_4$	402.2630	−2.2
aspochalasin D (**3**)[a,b]	384.253	$[M-H_2O+H]^+$	$C_{24}H_{34}NO_3$	384.2523	−2.7
asperphenamate (**4**)[a,c]	507.228	$[M+H]^+$	$C_{32}H_{31}N_2O_4$	507.2279	+0.1
N-benzoyl-L-phenylalaninol (**5**)[a,c]	256.133	$[M+H]^+$	$C_{16}H_{18}NO_2$	256.1328	+1.6
aspochalasin I[c]	416.240	$[M+H]^+$	$C_{24}H_{34}NO_5$	416.2437	+1.3
aspochalasin K[c]	434.289	$[M+H]^+$	$C_{25}H_{40}NO_5$	434.2906	+1.2
flavichalasin N[c]	434.253	$[M+H]^+$	$C_{25}H_{40}NO_5$	434.2906	+1.2
emeriphenolicin F[c]	734.459	$[M+H]^+$	$C_{44}H_{64}NO_8$	734.4632	+0.8
erythroskyrin[c]	456.235	$[M+H]^+$	$C_{26}H_{34}NO_6$	456.2386	+1.2
Aspergillus sp. NCA264					
diorcinol (**7**)[a,c]	229.086	$[M-H]^-$	$C_{14}H_{13}O_3$	229.0863	−3.1
sydonic acid (**8**)[a,c]	265.144	$[M-H]^-$	$C_{15}H_{21}O_4$	265.1442	−1.3
11-dehydrosydonic acid (**9**)[a,c]	263.129	$[M-H]^-$	$C_{15}H_{19}O_4$	263.1286	−1.1
(*S*)-10-hydroxysydonic acid[c]	281.139	$[M-H]^-$	$C_{15}H_{21}O_5$	281.1389	−1.9
Aspergillus sp. NCA257					
Asterriquinol D dimethyl ether (**10**)[a,b]	429.180	$[M+H]^+$	$C_{26}H_{25}N_2O_4$	429.1800	−2.1

[a]Isolated compound. [b]Manual annotation. [c]GNPS annotation. [d]Values from GNPS. [e]Values from UPLC-HRESIMS-MS/MS analysis.

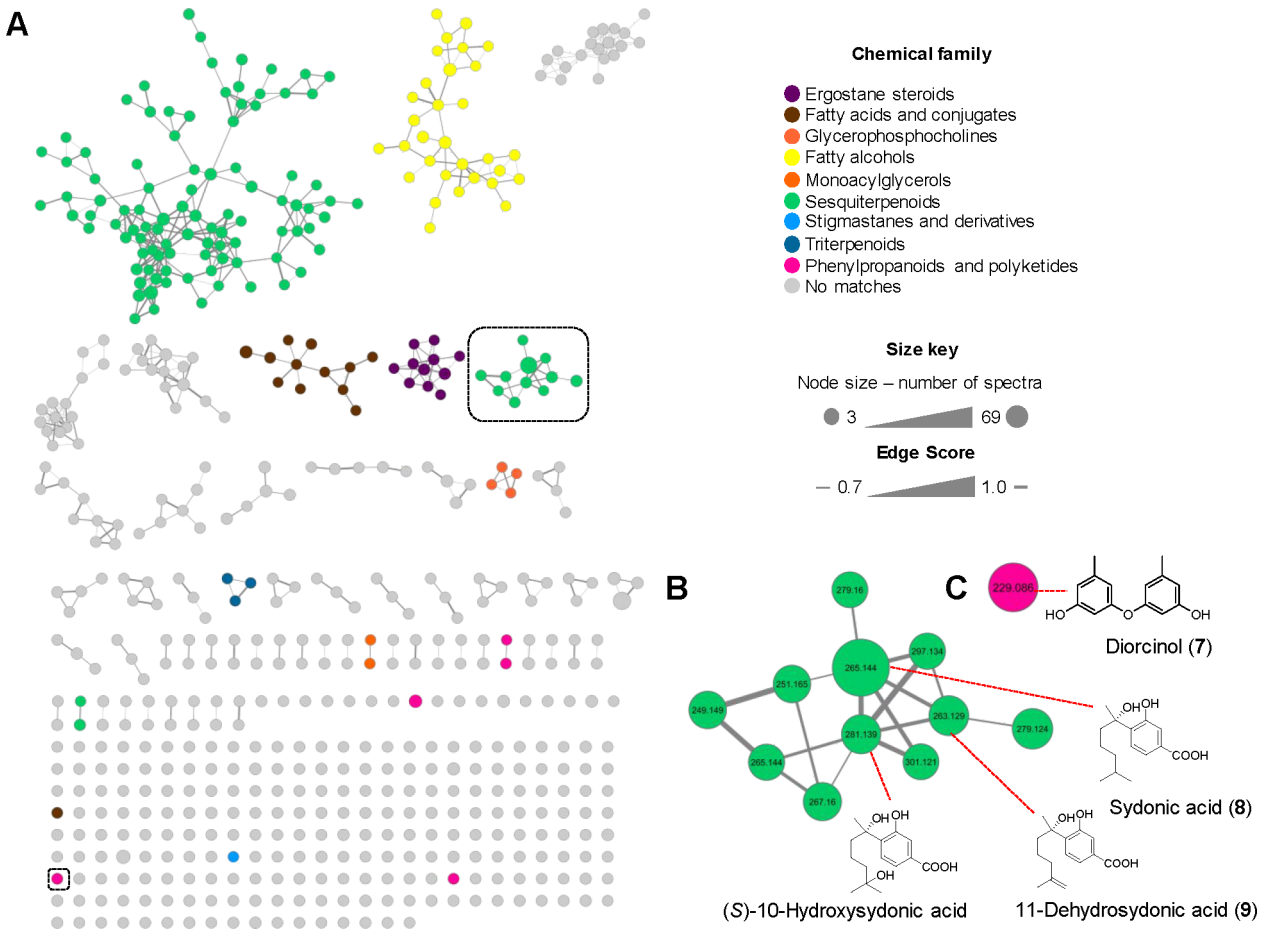

Figure 6. Metabolomic analysis of *Aspergillus* sp. NCA264. (A) FBMN and (B,C) selected clusters with nodes showing the compounds annotated by GNPS library search.

16, and by GNPS the annotation of a series of related phenylspyrodrimanes, including **15**, asperugin,[46] and stachybysbin A[47] (Figure 9 and Table 2).

The chemical study of the culture of *Cladosporium* sp. NCA273 did not yield any pure compound due to the scarcity of the organic extract obtained from the solid culture (poor growth in this medium). In addition, the MN analysis of this strain grouped the metabolite features into 77 nodes arranged in 6 clusters with >3 nodes per cluster, 8 with two nodes, and 63 singletons. No matches were found using the in-house

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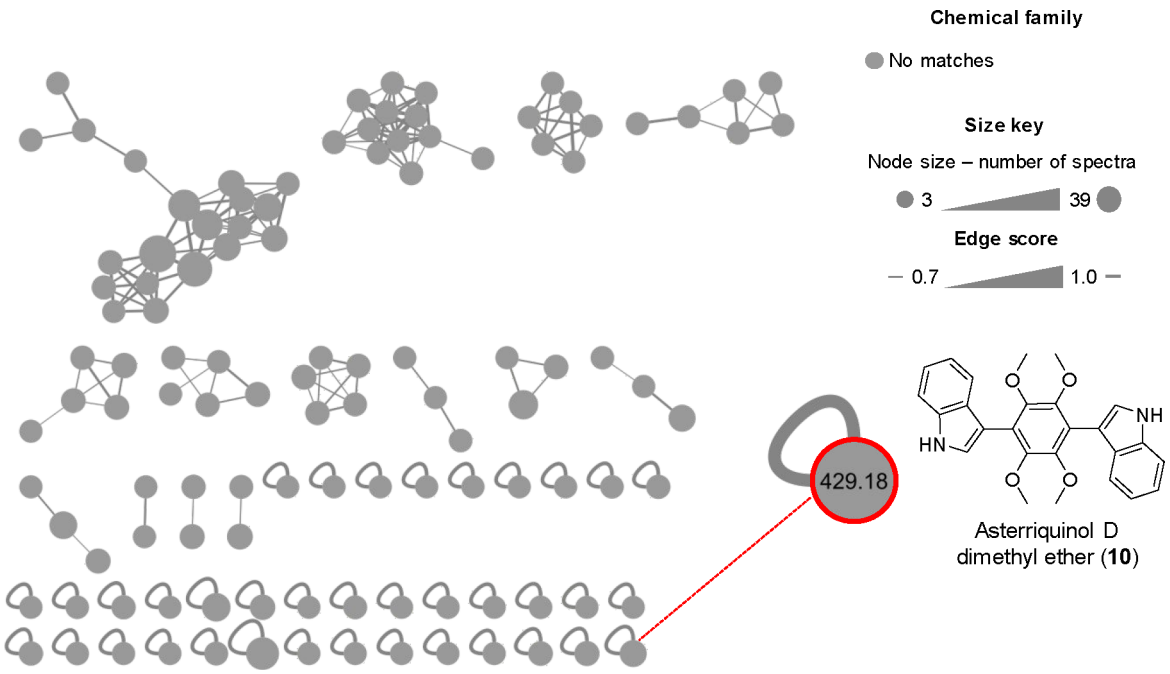

Figure 7. Metabolomic analysis of *Aspergillus* sp. NCA257 and selected node showing manually annotated **9** (red circle).

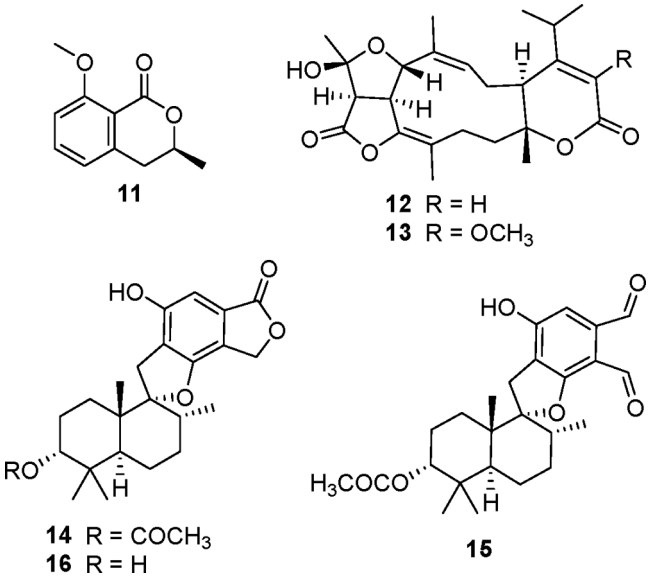

Figure 8. Isolated compounds from *Stachybotrys* sp. NCA252.

dereplication procedure nor the spectral library search in the GNPS, and only two chemical families (O-glycosyl compounds and glycerophosphocholines) were observed by FBMN (Figure S45).

***Aspergillus* spp. NCA257 and NCA276 and *Cladosporium* sp. NCA273 Coculture Analysis.** During the isolation process of the fungal strains from the cenote sediment samples, a series of antagonistic interactions between *Aspergillus* spp. NCA257 and NCA276 and *Cladosporium* sp. NCA273 were observed (Figure 10). It is known that coculture fermentation can trigger the expression of silent biosynthetic gene clusters to produce new secondary metabolites, increase the amounts of others, or in some cases inhibit the production of some compounds.[48−50] This strategy was thus employed to analyze the chemical variation of these strains when cocultivated in solid media (Supporting Information).

High-performance liquid chromatography (HPLC) analysis of the organic extract of the coculture of *Aspergillus* sp. NCA257 and *Cladosporium* sp. NCA273 revealed the presence of penicillic acid (**17**),[51] 5,6-dihydropenicillic acid (**18**),[52] and 3-isobutyl-6-(1-hydroxy-2-methylpropyl)-pyrazin-2(1*H*)-one (**19**)[53] (Figure 11). Compound **17** was not isolated from the monoculture of *Aspergillus* sp. NCA257 due to its low abundance. However, the induction of **17**−**19** by coculture allowed its isolation and structural elucidation (Supporting Information). Interestingly, **17** abundance (AUC by HPLC analysis) was 250% times higher in the coculture compared to the monoculture of *Aspergillus* sp. NCA257, whereas **10** was totally suppressed. On the other hand, the analysis of the *Aspergillus* spp. NCA257 and NCA276 coculture revealed similar results than those of NCA257 and NCA273 coculture, that is, **17**−**19** were induced in the coculture, and the aspochalasins **1** and **3** were suppressed (Figure 12).

The MN analysis by GNPS of the cocultures of NCA257/NCA273 and NCA257/NCA276, and the single-cultures of all three strains consisted of 1380 nodes, which were grouped into 114 clusters with >2 nodes per cluster (Figure S46). Interestingly, the MN showed a major contribution from strain NCA257 (*Aspergillus* sp.) in the clusters of the chemical features (Figure S46). This agrees with the HPLC profiles (Figures 11 and 12), where the production of the penicillic acids **17** and **18**, and the pyrazinone **19** suppresses the biosynthesis of the main metabolites from the other strains.[54] Finally, aspochalasin J, aspochalasinol A, and alterporriol B were annotated by GNPS in MN (Figure S46 and Table S4, Supporting Information).

Biological Activity of Pure Compounds. The antibacterial activity of the isolated compounds was assessed against a panel of ESKAPE pathogens using the microdilution assay[55,56] at 100 μg/mL or 10 μg/mL (Table 3). From these, the aspochalasin **3**, the amino acid ester **4**, the bis-indolyl

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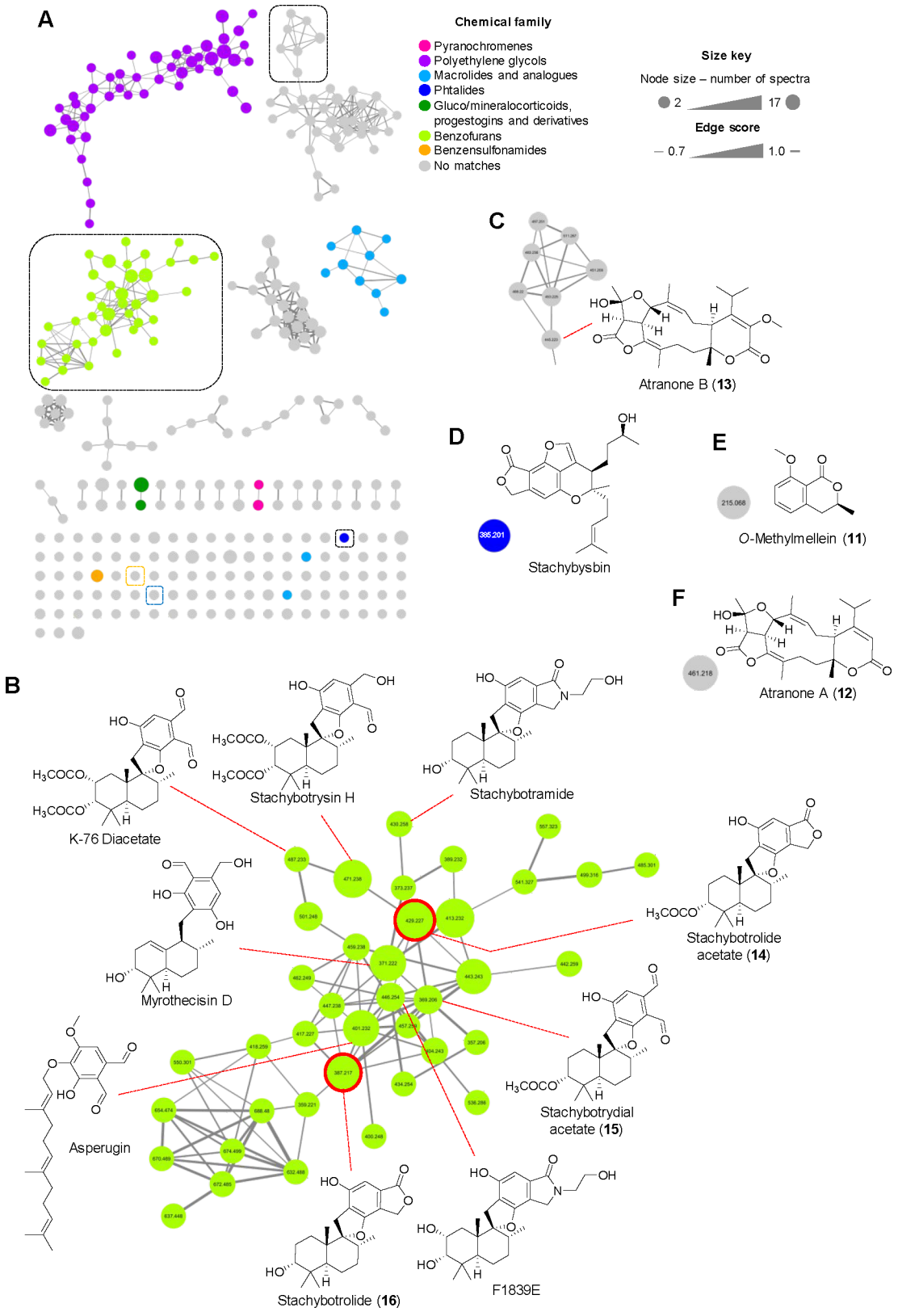

Figure 9. Metabolomic analysis of *Stachybotrys* sp. NCA252. (A) FBMN and (B–F) selected clusters with nodes showing the compounds manually annotated (red circles) and by GNPS library search.

benzenoid **10**, and the phenylspirodrimanes **14–16** showed high activity (>50% inhibition) against MSSA and MRSA strains with **14** as the most potent metabolite (>81% inhibition at test concentration of 10 μg/mL). None of the tested compounds were active against *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella aerogenes* ATCC 13048, *K. pneumoniae* ATCC 700603, *Acinetobacter baumannii* ATCC 17978, and the multidrug-resistant *A. baumannii* clinical strain A564.[57]

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Table 2. Chemical Annotation of Metabolites from *Stachybotrys* sp. NCA252

compound	observed ion (m/z)[d]	adduct	molecular formula	exact mass (m/z)[e]	mass accuracy (ppm)
O-methylmellein (**11**)[a,b]	215.068	$[M+Na]^+$	$C_{11}H_{12}O_3Na$	215.0675	+1.7
atranone A (**12**)[a,b]	461.218	$[M+HCOO]^-$	$C_{25}H_{33}O_8$	461.2182	+0.2
atranone B (**13**)[a,b]	445.223	$[M-H]^-$	$C_{25}H_{33}O_7$	445.2226	−1.3
stachybotrolide acetate (**14**)[a,b]	429.227	$[M+H]^+$	$C_{25}H_{33}O_6$	429.2272	+0.1
stachybotrydial acetate (**15**)[a]	369.206	$[M-AcO+H]^+$	$C_{23}H_{29}O_4$	369.2061	+0.2
stachybotrolide (**16**)[a,b]	387.217	$[M+H]^+$	$C_{23}H_{31}O_5$	387.2160	−1.6
stachybotramide[c]	430.258	$[M+H]^+$	$C_{25}H_{36}NO_5$	430.2593	+1.2
K-76 diacetate[c]	487.233	$[M+H]^+$	$C_{27}H_{35}O_8$	487.2332	+1.1
stachybotrysin H[c]	471.238	$[M-H_2O+H]^+$	$C_{27}H_{35}O_7$	471.2383	+1.2
myrothecisin D[c]	371.222	$[M-H_2O+H]^+$	$C_{23}H_{31}O_4$	371.2222	+1.4
F1839E[c]	446.254	$[M+H]^+$	$C_{25}H_{36}NO_6$	446.2542	+1.1
asperugin[c]	401.232	$[M+H]^+$	$C_{24}H_{33}O_5$	401.2328	+1.4
stachybysbin A[c]	385.201	$[M+H]^+$	$C_{23}H_{29}O_5$	385.2015	+1.4

[a]Isolated compound. [b]Manually annotation. [c]GNPS annotation. [d]Values from GNPS. [e]Values from UPLC-HRESIMS-MS/MS analysis.

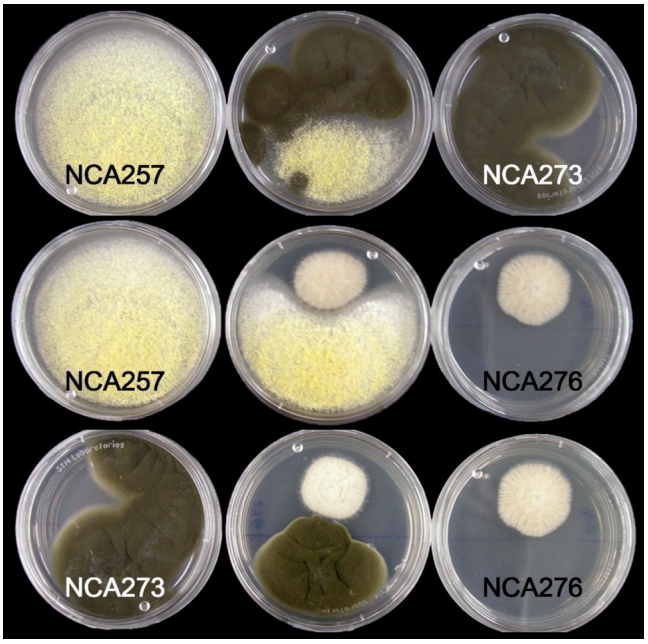

Figure 10. Fungal interaction between *Aspergillus* spp. NCA257 and NCA276 and *Cladosporium* sp. NCA273 in PDA plates.

Finally, **7**, **9**, and **19** were not tested due to the scarcity of the isolated material.

In addition, the anti-*mycobacteria* activity of the isolated compounds was assessed using the microplate Alamar blue (MABA) and low oxygen recovery (LORA) assays[58,59] against tuberculous (TB) and nontuberculous mycobacterias (NTM), respectively, and a cytotoxicity assay against Vero cell line (ATCC CCL-81)[60] (Table 4). Compounds **3**, **4**, **7**, **14**, **16**, and **17** showed >98% growth inhibition of TB at 50 μg/mL and CC_{50} values >35 μM against Vero cells. Moreover, **3**, **4**, **7**, **14**, and **16**, displayed anti-*M. avium* activity with MIC values of 22.6, 37.0, 20.0, 11.6, and 17.0 μM, respectively, and **7**, **14**, and **16** against *M. marinum* with MIC values of 47.2, 23.1, and 47.0 μM, respectively. Compounds **2** and **9** were not tested in these assays due to the paucity of the isolated material.

CONCLUSIONS

In summary, this work represents the first contribution to the chemical diversity and biology of fungi from sediments in a cenote of the Yucatan peninsula. Strains *Aspergillus* (NCA257, NCA264, and NCA276), *Stachybotrys* (NCA252), and *Cladosporium* (NCA273) were isolated from sediments' samples collected in the cenote Tza Itzá and taxonomically identified by molecular sequencing of the ITS rDNA. From this, several isolated compounds showed significant activity against MSSA, MRSA, other ESKAPE pathogens and mycobacterial strains and could be further studied for the development of potential drug leads. The absolute configuration of new 17-deoxy-aspergillin PZ (**1**) was established via single-crystal X-ray crystallography. Finally, cocultures and metabolomics analysis revealed that communication between the strains is needed to produce metabolites that were not present in the monocultures. Overall, this work uncovers the chemical and biological potential of understudied fungal strains to produce biosynthetic bioactive natural products.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations, and UV data were measured using a Rudolph Research Autopol III polarimeter (Rudolph Research Analytical), and a Varian Cary 100 Bio UV−vis spectrophotometer (Varian Inc.), respectively. NMR experiments were conducted in $CDCl_3$ or methanol-d_4, using a JEOL ECA-600 spectrometer (JEOL Ltd.), a Varian VNMRS 400 (Varian Inc.), or a Bruker Avance III 400 MHz (Bruker BioSpin Corp.). HRESIMS data were acquired using a Q Exactive Plus system (Thermo Fisher Scientific), equipped with an electrospray ionization source with an HCD cell. Data were collected in both positive and negative modes via direct injection or through an Acquity UPLC system (Waters Corp.) using a BEH C_{18} column (50 × 2.1 mm i.d., 1.7 μm; Waters Corp.) with a gradient solvent system from 15:85 to 100:0 CH_3CN-0.1% aqueous formic acid for 10 min at a flow rate of 0.3 mL/min. Analytical and preparative HPLC were carried out on a Waters HPLC system equipped with a 2535 quaternary pump, a 2707 autosampler, and 2998 PDA and 2424 ELSD detectors, using Gemini C_{18} and Kinetex C_{18} columns (5 μm,110 Å, 250 × 4.6 mm i.d. and 5 μm, 110 Å, 250 × 21.2 mm i.d.; Phenomenex) for analytical and preparative runs, respectively. Data acquisition and management were performed with the Empower 3 software (Waters Corp.). Flash chromatography was conducted on a CombiFlash Rf+ Lumen system (Teledyne Technologies Inc.) equipped with PDA and ELSD detectors using RediSep Rf Gold Si-gel columns (Teledyne Technologies Inc.). Reagent-

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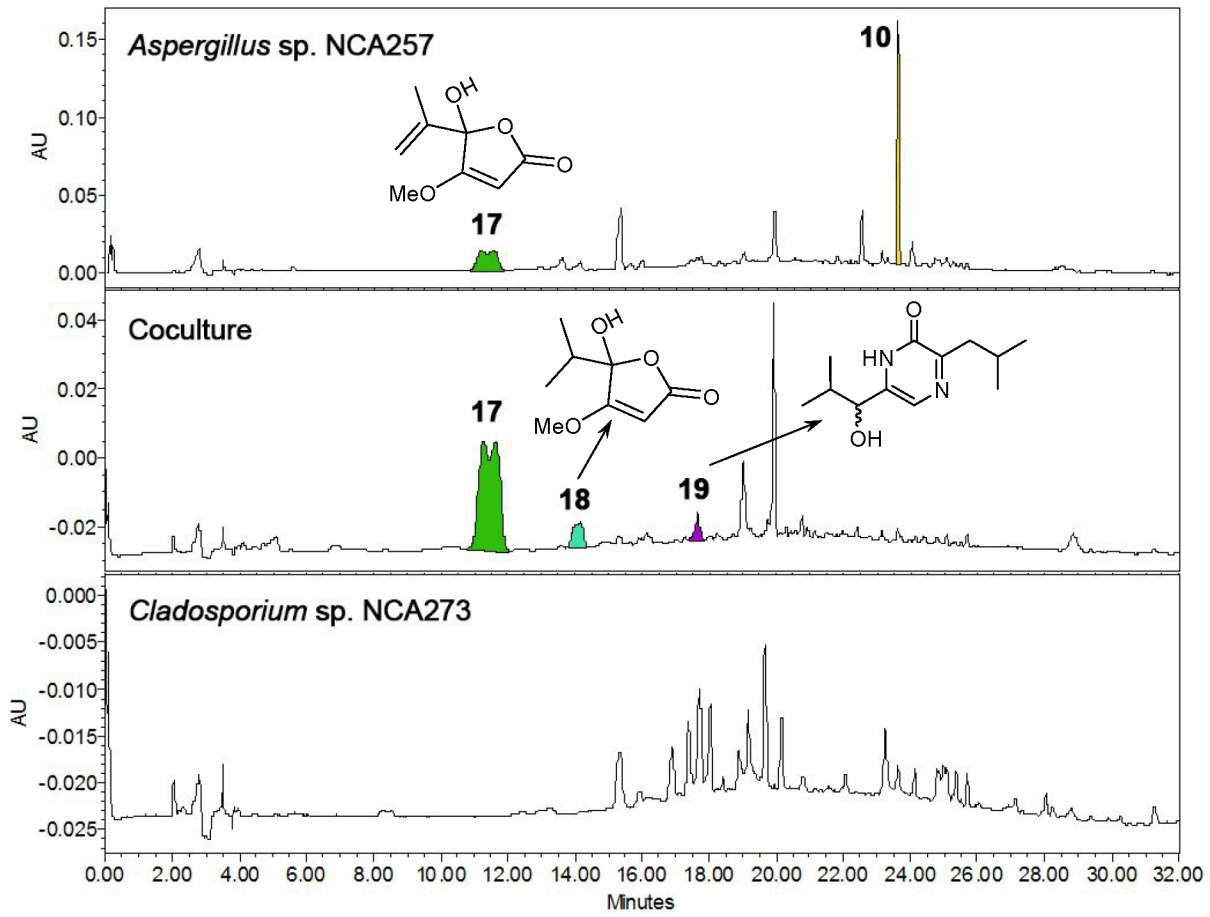

Figure 11. HPLC traces (PDA UV λ = 254 nm) of monoculture extracts of *Aspergillus* sp. NCA257 and *Cladosporium* sp. NCA273 and the coculture extract.

grade chloroform, *n*-hexane, and methanol, and HPLC- and MS-grade acetonitrile, methanol, and water were purchased from J.T. Baker (Avantor Performance Materials). Deuterated NMR solvents were acquired from Cambridge Isotope Laboratories, Inc.

Fungal Strains Isolation and Identification. The fungi of interest were isolated from sediment samples collected from cenote *Tza Itzá* (20°43′50.27″ N, 89°27′56.82″ W), in the state of Yucatán, Mexico in 2018. The cenote dimensions are a maximum depth of 40 m, water mirror of 7.6 × 10.5 m, and dry cave of 30 × 8 × 5 m (L × W × H). The isolation of the strains was made in A1 10% media with peptone (0.4 g), starch (1.0 g), yeast extract (0.2 g), supplemented with rifampicin (5 μg/mL) and cycloheximide (0.1 mg/mL) to reduce the growth of Gram-negative bacteria as previously described.[10] The fungal cultures are maintained at −80 °C at the microbial culture collection from Unidad de Química en Sisal, Facultad de Química, UNAM, in Yucatan, Mexico. A copy of each strain used for the chemical studies is also maintained at the Departamento de Farmacia, Facultad de Química, UNAM, Mexico. Axenic cultures of strains NCA252, NCA257, NCA264, NCA273, and NCA276 were subjected to molecular identification by ITS sequencing and BLAST search with the RefSeg database using the methodologies reported.[61,62] For designation of taxonomic names, the results of the ITS BLAST search using GenBank were interpreted with caution using modification of outlined criteria.[63] Since we sequenced only the ITS region, we chose a rather conservative approach and made identifications only to the genus level even when BLAST sequence homology was ≥99%. Sequences for each strain were deposited in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) and given the GenBank Data Base accession numbers indicated in (Table S1 Supporting Information).

Fungal Cultivation, Extraction, and Isolation and Characterization of Pure Compounds. The methods utilized to culture the fungal strains (mono and cocultures) to prepare their organic extracts and to isolate and structurally elucidate compounds **1**−**19** from the fungi followed well-established protocols[64−67] and are detailed in the Supporting Information.

17-Deoxy-aspergillin PZ (1). Colorless solid; $[\alpha]_D^{18.1}$ + 7.5 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 322 (3.28), 285 (3.34) nm; ^1H and ^{13}C NMR, see Table S3 on Supporting Information; HRESIMS *m*/*z* 386.2682 $[M + H]^+$ (calcd. for $C_{24}H_{36}NO_3$, 386.2695).

X-ray Crystallographic Analysis of 17-deoxy-aspergillin PZ (1). Suitable X-ray quality single crystals for 17-deoxy-aspergillin PZ (**1**) were successfully obtained from MeOH−$CHCl_3$−H_2O (20:1:1) recrystallization mixture. The crystallographic data and data collection parameters are mentioned in Table S2. All reflection intensities were measured at 100(2) K using a Gemini R diffractometer (equipped with Atlas detector) with CuKα radiation (λ = 1.54178 Å) under the program CrysAlisPro (Version CrysAlisPro 1.171.38.43f, Rigaku OD, 2015). The refinement

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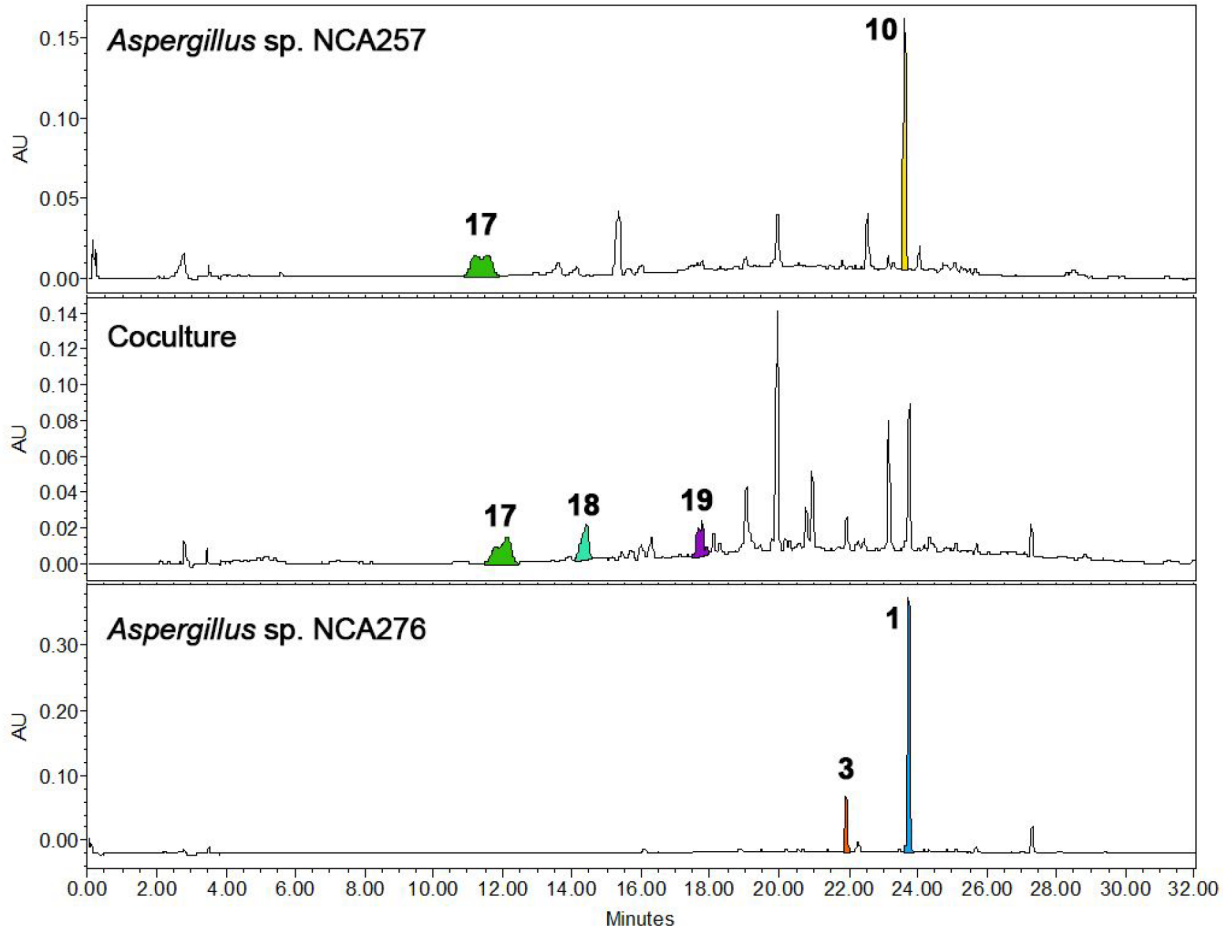

Figure 12. HPLC traces (PDA UV λ = 254 nm) of monoculture extracts of *Aspergillus* spp. NCA257 and NCA276 and the coculture extract.

Table 3. Antimicrobial Activity of Isolated Compounds against ESKAPE Bacteria at 100 μg/mL

	growth inhibition (%)				
compound	VSEF[e]	VREF[e]	MSSA[e]	MRSA[e]	EC[e]
17-deoxy-aspergillin PZ (**1**)			11.8	27.7	28.1
aspergillin PZ (**2**)				23.0	9.0
aspochalasin D (**3**)	37.2	23.8	81.4	87.2	
asperphenamate (**4**)			10.4	62.9	22.5
N-benzoyl-L-phenylalaninol (**5**)			13.3	50.7	
2-*O*-methylbutyrolactone II (**6**)					
sydonic acid (**8**)				34.0	11.9
asterriquinol D dimethyl ether (**10**)			50.9	62.3	48.2
O-methylmellein (**11**)					16.2
atranone A (**12**)	13.0				6.0
atranone B (**13**)					12.2
stachybotrolide acetate (**14**)[d]			81.0	92.9	21.5
stachybotrydial acetate (**15**)			82.7	89.3	18.3
stachybotrolide (**16**)			82.2	91.2	23.7
penicillic acid (**17**)			80.4	51.4	
5,6-dihydropenicillic acid (**18**)	38.3				
MIC positive control in μg/mL.	3.75[a]	25.0[a]	200.0[b]	2.5[a]	0.5[c]

[a]Vancomycin - Inactive at 100 μg/mL. [b]Ampicillin - Inactive at 100 μg/mL. [c]Gentamicin - Inactive at 100 μg/mL. [d]Tested at 10 μg/mL. [e]VSEF, vancomycin-susceptible *E. faecalis* ATCC 29212; VREF, vancomycin-resistant *E. faecalis* ATCC 51299; MSSA, methicillin-susceptible *S. aureus* ATCC 25923; MRSA, methicillin-resistant *S. aureus* ATCC 43300; and EC, *E. cloacae* ATCC 700323.

of cell dimensions and data reduction were performed using the most recent version of the program (viz. CrysAlisPro 1.171.40.53, Rigaku OD, 2019). The structure was solved with the program SHELXT-2018/2 and was refined on F^2 by the full-matrix least-squares method using SHELXL-2018/3.[68] An empirical absorption correction was applied using CrysAlisPro 1.171.40.53.[69] Non-hydrogen atoms were refined anisotropically. In the refinement, hydrogens attached to carbon, were

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Table 4. Anti-*mycobacteria* and Cytotoxic Activities of Isolated Compounds

	M. tuberculosis H37Rv		*M. abscessus* ATCC 19977	*M. chelonae* ATCC 35752	*M. marinum* ATCC 927	*M. avium* ATCC 15769	*M. kansasii* ATCC 12478	
compound	MABA[a]	LORA[b]			MABA[c]			Vero cell ATCC CCL-81[d]
17-deoxy-aspergillin PZ (**1**)	19 (ND)	ND	ND	ND	ND	ND	ND	ND
aspochalasin D (**3**)	101 (39.5)	>50	>50 (0)	>50 (17)	>50 (31)	22.55	>50 (16)	37.5
asperphenamate (**4**)	101 (16.6)	>50	>50 (0)	>50 (6)	>50 (22)	37.00	>50 (0)	>50
N-benzoyl-L-phenylalaninol (**5**)	20 (ND)	ND	ND	ND	ND	ND	ND	ND
2-*O*-methylbutyrolactone II (**6**)	83 (ND)	ND	ND	ND	ND	ND	ND	ND
diorcinol (**7**)	100 (47.9)	>50	>50 (0)	>50 (12)	47.24	19.98	>50 (0)	39.9
sydonic acid (**8**)	30 (ND)	ND	ND	ND	ND	ND	ND	ND
asterriquinol D dimethyl ether (**10**)	13 (ND)	ND	ND	ND	ND	ND	ND	ND
O-methylmellein (**11**)	22 (ND)	ND	ND	ND	ND	ND	ND	ND
atranone A (**12**)	29 (ND)	ND	ND	ND	ND	ND	ND	ND
atranone B (**13**)	33 (ND)	ND	ND	ND	ND	ND	ND	ND
stachybotrolide acetate (**14**)	99 (48.3)	>50	>50 (0)	>50(0)	23.15	11.63	>50(0)	38.2
stachybotrydial acetate (**15**)	38 (ND)	ND	ND	ND	ND	ND	ND	ND
stachybotrolide (**16**)	103 (49.4)	>50	>50 (0)	>50 (0)	47.02	16.92	>50(0)	39.7
penicillic acid (**17**)	98 (49.5)	>50	ND	ND	ND	ND	ND	ND
5,6-dihydropenicillic acid (**18**)	42 (ND)	ND	>50 (0)	>50 (73)	>50 (58)	>50 (53)	>50(0)	35.4
3-isobutyl-6-(1-hydroxy-2-methylpropyl) -pyrazin-2(1H)-one (**19**)	23 (ND)	ND	ND	ND	ND	ND	ND	ND
MIC Rifampicin (μg/mL)	100 (0.03)	0.08	>8.0	4.25	0.09	0.05	0.41	>8.0

[a]% Inhibition at 50 μg/mL (MIC μM). [b]MIC μM. [c]MIC μM (% Inhibition). [d]CC_{50}, cytotoxic concentration to 50% inhibition of the cell line. ND, not determined since % inhibition of *M. tuberculosis H37Rv* using MABA was below 90%.

treated as riding atoms using SHELXL default parameters, while those attached to nitrogen were located with electron difference maps. The crystallographic data of **1** in CIF format was deposited in the Cambridge Structural Database (CSD) (CCDC no. 2142221).

Biological Activity Assays. Pure compounds were tested *in vitro* for antibacterial activity using the Clinical and Laboratory Standards Institute (CLSI) broth dilution standard method.[55,56] Target bacteria used in the assays belong to the ESKAPE group: vancomycin-susceptible *Enterococcus faecalis* ATCC 29212 (VSEF), vancomycin-resistant *E. faecalis* ATCC 51299 (VREF), methicillin-susceptible *Staphylococcus aureus* ATCC 25923 (MSSA), methicillin-resistant *S. aureus* ATCC 43300 (MRSA), *Klebsiella aerogenes* ATCC 13048, *K. pneumoniae* ATCC 700603, *Acinetobacter baumannii* ATCC 17978, multidrug-resistant *A. baumannii* strain 564 (clinical isolated),[57] *Pseudomonas aeruginosa* ATCC 27853, *and Enterobacter cloacae* ATCC 700323. The compounds were dissolved in DMSO to obtain a stock solution and then tested at a final concentration of 100 μg/mL or 10 μg/mL. The bioassays were carried out in 96-well plates in triplicate at concentrations of pure compound. In addition, pure compounds were also tested against *M. tuberculosis* H37Rv (MT) strain under both aerobic (replicating) and anaerobic (nonreplicating; NRMT) conditions using MABA and LORA assays.[58] Compounds with activity >90% of inhibition of the growth of MT in MABA were further tested against *M. abscessus* ATCC 19977, *M. chelonae* ATCC 35752, *M. marinum* ATCC 927, *M. avium* ATCC 15769, and *M. kansasii* ATCC 12478, using MABA[58] and a cytotoxicity assay against Vero cell line ATCC CCL-81.[60] For the latter, 0.6 mM of resazurin was used and the absorbance was recorded at 530 nm (excitation) and 590 nm (emission). Positive controls for all assays are indicated in Tables 3 and 4.

Molecular Networking and Metabolomics Analysis. Each extract from the mono and cocultures was analyzed by LC-HRMS-MS/MS using a previously described methodology.[66,67] Raw data were converted to mzML format using the ProteoWizard tool MsConvert (version 3.0.20239) and the resulting files uploaded to the Global Natural Products Social (GNPS; https://gnps.ucsd.edu) Web server using the FTP Server Version 5.17.16. Molecular networks were generated following the workflow previously published.[31,32] Data was filtered by removing all MS/MS fragment ions within ±17 Da of the precursor *m*/*z*. The precursor ion mass tolerance was set to 0.01 Da and a MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.7 and more than five matched peaks. Furthermore, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches between network spectra and library spectra were required to have a score above 0.7 and at least five matched peaks. GNPS MolNetEnhancer and Dereplicator+ tools were applied for chemical classification.[33,70] Molecular networks were visualized with Cytoscape 3.8.1.[71] Finally, manual dereplication was assessed using UV-absorption maxima and HRMS-MS/MS data against MS/MS data of **1**−**19** and by comparison with those reported in the Dictionary of Natural Products,[72] SciFinder,[73] and an in-house mycotoxins database. The annotation of isolated compounds was at confidence level 1, according to the metabolomics standards initiative and exact mass accuracy <5 ppm.[74]

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00544.

Methods for fungal cultivation, extraction, and isolation and characterization of pure compounds; identity of fungal isolates; NMR and MS spectra of **1**−**19**; crystallographic data of **1**; MN and chemical annotation of cocultures (PDF)

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Author Contributions

This work was part for the Ph.D. thesis of C.A.F.-H. from the Posgrado en Ciencias Qumicas, UNAM. C.A.F.-H. and M.F. designed the experiments. C.A.F.-H., F.S.T.K., L.F.-B., A.P.-D., B.W., R.M., M.Q., S.G.F., R.V.-S., A.M.-C., M.A.L.-L., and H.A.R. performed the experiments. C.A.F.-H., A.P.-D., S.H., S.G.F., H.A.R., R.G.-C., and M.F. analyzed the data and revised the manuscript. C.A.F.-H. and M.F. wrote, reviewed, and edited the manuscript. All authors have read and agreed to the revised version of the manuscript.

Notes

The authors declare no competing financial interest.

LC-MS/MS data can be accessed at MassIVE (accession no. MSV000088691; accessed on January 26, 2022). The molecular network data set of fungal coculture species *Aspergillus* spp. NCA257, NCA276 and *Cladosporium* sp. NCA273 can be accessed at MassIVE (accession no. MSV000088690; accessed on January 26, 2022). The molecular network of *Stachybotrys* sp. NCA252 at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=2e6ed82df2854ee481908c5e7be883e0 (MolNetEnhancer analysis; accessed on January 26, 2022), molecular network of *Aspergillus* sp. NCA257 at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=2aea96c6e47746e28349aa3241c8d065 (MolNetEnhancer analysis; accessed on January 26, 2022), molecular network of *Aspergillus* sp. NCA264 at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=63f8482f83c94d3ea4b34910a972f170 (MolNetEnhancer analysis; accessed on January 26, 2022), molecular network of *Cladosporium* sp. NCA273 at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=5f13c0f6339742879130611c389af5da (MolNetEnhancer analysis; accessed on January 26, 2022) and molecular network of *Aspergillus* sp. NCA276 at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ee9f321cb43d4e1da6f38fde4cfa696b (MolNetEnhancer analysis; accessed on January 26, 2022). Molecular network of fungal coculture species *Aspergillus* spp. NCA257, NCA276 and *Cladosporium* sp. NCA273 at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=66c153a4faa4418485b252df22c99023 (accessed on January 26, 2022).

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