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Article

OSMAC Approach and Cocultivation for the Induction of Secondary Metabolism of the Fungus *Pleotrichocladium opacum*

Víctor Rodríguez Martín-Aragón, Mónica Trigal Martínez, Cristina Cuadrado, Antonio Hernández Daranas,* Antonio Fernández Medarde, and José M. Sánchez López*

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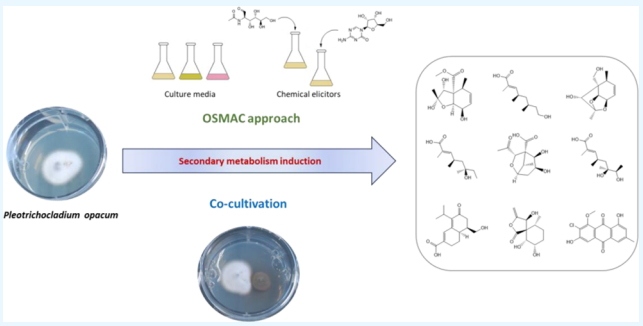
ABSTRACT: The application of an OSMAC (One Strain−Many Compounds) approach on the fungus *Pleotrichocladium opacum*, isolated from a soil sample collected on the coast of Asturias (Spain), using different culture media, chemical elicitors, and cocultivation techniques resulted in the isolation and identification of nine new compounds (**8**, **9**, **12**, **15**−**18**, **20**, **21**), along with 15 known ones (**1**−**7**, **10**, **11**, **14**, **19**, **22**−**25**). Compounds **1**−**9** were detected in fungal extracts from JSA liquid fermentation, compounds **10**−**12** were isolated from a solid rice medium, whereas compounds **14** and **15** were isolated from a solid wheat medium. Addition of 5-azacytidine to the solid rice medium caused the accumulation of compounds **16**−**18**, whereas adding *N*-acetyl-D-glucosamine triggered the production of two additional metabolites, **19** and **20**. Finally, cocultivation of the fungus *Pleotrichocladium opacum* with *Echinocatena* sp. in a solid PDA medium led to the production of five additional natural products, **21**−**25**. The structures of the new compounds were elucidated by HRESIMS and 1D and 2D NMR as well as by comparison with literature data. DP4+ and mix-*J*-DP4 computational methods were applied to determine the relative configurations of the novel compounds, and in some cases, the absolute configurations were assigned by a comparison of the optical rotations with those of related natural products.



INTRODUCTION

There is an urgent need to discover new drug candidates due to the increase in severe health issues such as cancer, neurodegenerative pathologies, and antibiotic resistance. In recent years, there has been a renewed interest in exploring microbial sources in the search for new biologically active natural products, which has been driven by the advancement of cultivation and molecular biology techniques.[1,2]

In our search for new biologically active fungal secondary metabolites, we investigated the fungus *Pleotrichocladium opacum* isolated from a soil sample collected on the coast of Asturias (Spain). LCMS profiling of a *Pleotrichocladium opacum* extract revealed up to 10 different compounds, and some of them did not dereplicate against the Biomar Microbial Technologies pure compound database. Fungi of this genus have been reported to produce several cytotoxic secondary metabolites, such as trichocladinols A−C.[3]

In this study, an OSMAC (One Strain−Many Compounds) approach and cocultivation for the induction of cryptic secondary metabolites of the fungus *Pleotrichocladium opacum* were followed. The OSMAC approach has been shown to be a simple and powerful tool to promote the production of different secondary metabolites, potentially bioactive, by altering simple culture parameters, such as variation on medium composition or addition of epigenetic modifiers (chemical elicitors). Furthermore, in contrast to traditional screening processes involving the cultivation of a single microbial strain (axenic culture) for the discovery of new bioactive compounds, the introduction of coculture techniques presents new opportunities for the activation of cryptic biosynthetic pathways.[4−8] For this purpose, the fungus *Pleotrichocladium opacum* was cultivated on different culture media, both liquid and solid, and cocultured with the fungus *Echinocatena* sp., a marine fungus with a very different ecological origin, isolated from a gorgonian sample collected in Equatorial Guinea at a depth of 23 m in a reef close to Bioko Island

Herein we report on the details of the isolation, structure elucidation, and bioactivity assays of the new natural products discovered.

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RESULTS AND DISCUSSION

Fermentation of the microorganism was carried out under standard conditions (JSA medium). As a result, nine natural products (**1**−**9**), including two new compounds (**8** and **9**), were isolated, and their structures were elucidated (Figure 1).

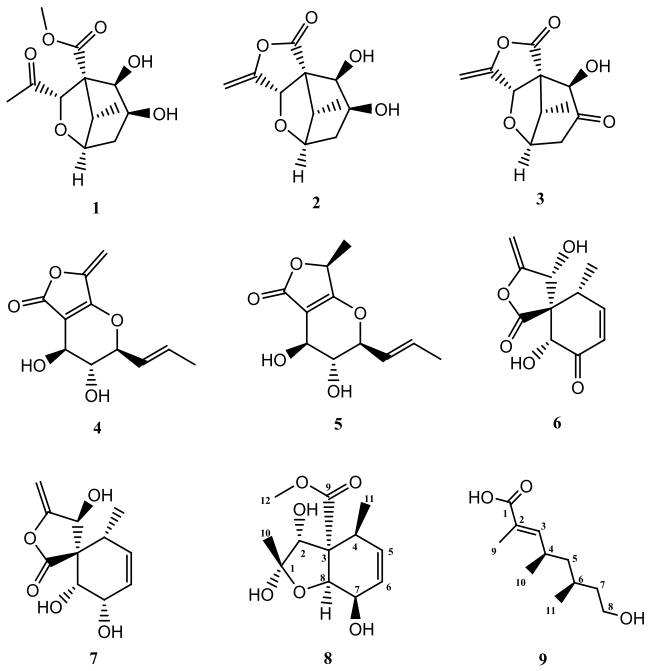

Figure 1. Secondary metabolites isolated from *Pleotrichocladium opacum* (JSA medium culture).

Compounds **1**−**7** were identified as the already known massarilactones C (**1**),[9] A (**2**),[10] E (**3**),[11] H (**4**),[12,13] and B (**5**)[10] and massarigenins C (**6**) and A (**7**)[14], by comparison of their HRESIMS and 1D and 2D NMR spectra with those described in the literature.

Compound **8** was isolated as a colorless solid. Its molecular formula was determined as $C_{12}H_{18}O_6$ (four degrees of unsaturation) based on HRESIMS and NMR data. Its ^{1}H and ^{13}C NMR spectra (Table 1) suggested the presence of two methyl groups, one of them forming a CH_3CH- moiety; a methoxy group; an ester carbonyl (δ_C 177.0); three oxymethine hydrogens; two sp^3 quaternary carbons, one of them hemiacetalic (δ_C 56.8 and 102.0, respectively); a 1,2-disubstituted olefin; and three exchangeable hydrogens (δ_H 2.49, 4.03, and 4.20). The remaining two degrees of unsaturation indicated that **8** is a bicyclic compound.

Table 1. NMR Data of Compound 8 in $CDCl_3$ at 400 (^{1}H) and 100 (^{13}C) MHz

no.	δ_H (J in Hz)	δ_C, type	gHMBC	gCOSY
1		101.9, C		
2	3.57, (d, 10.0)	80.2, CH	C-4, 9	2-OH
3		56.7, C		
4	2.55, m	33.0, CH		5, 6, 11
5	5.54, (dt, 2.9, 9.6)	131.5, CH	C-3, 4, 11	4, 6, 7
6	5.88, (dm, 9.6)	133.8, CH	C-4	4, 5, 7, 8
7	4.11, m	68.3, CH		5, 6, 8, 7-OH
8	4.63, (dd, 1.5, 4.3)	83.1, CH	C-4, 6, 7, 9	6, 7
9		176.8, C		
10	1.40, s	23.2, CH_3	C-1, 2	
11	1.25, (d, 7.1)	15.8, CH_3	C-3, 4, 5	4
12	3.84, s	53.3, CH_3	C-9	
1-OH	4.20, s			
2-OH	4.03, (d, 10.0)			2
7-OH	2.49, (d, 10.4)			7

Analysis of the gCOSY (COrrelation SpectroscopY) spectrum allowed the assignment of two isolated spin systems corresponding to C4-C8/C11/7-OH and C2-2-OH, which, in conjunction with gHMBC correlations of H-8 to C-4 and of H-11 and H-5 to quaternary carbon C-3, established a pentasubstituted cyclohexene ring. Furthermore, gHMBC correlations of H-2 and H-8 to the ester carbonyl C-9, of H_3-10 to C-2 and the hemiacetalic carbon C-1, and of H-2 to C-4, in conjunction with the molecular formula of compound **8** and the chemical shifts of H-8 and C-8, suggested that C-1 must be linked through an ether bridge to C-8, establishing a tetrahydrofuran ring. Once the planar structure was determined, elucidation of the stereochemistry was faced. This task is generally tackled using spatial information obtained from the NOESY experiment. However, it is relatively common in natural product chemistry that the lack of a sufficient amount of sample prevents obtaining clear data from such experiment due to its low sensitivity. As an alternative, the use of quantum mechanical calculations to unravel the information encoded in NMR chemical shifts, also known as QM-NMR methods, is currently one of the most popular strategies for this purpose as they are becoming a quicker and, in some cases, even more reliable approach than the total synthesis approach.[15,16] QM-NMR approaches have been successfully used in structure elucidation of very complex compounds.[17−19] Although several methods are available, those based on Bayes' theorem, such as the original DP4 probability or the more elaborated DP4+ and *J*-DP4 methods, stand out from the rest.[20−23] All of them compare calculated chemical shifts versus the experimental data, obtaining the probability of a particular candidate to be correct.[24] Selection of the most appropriate method should consider the structure of the studied methods as well as the availability of useful coupling constant values.[25,26] Whereas the use of appropriate $^3J_{HH}$ values will fix conformations in the right position and will speed up all necessary calculations as in the *J*-DP4 methods, DP4+ needs less experimental data but consumes more computational time.[27,28] In summary, the chosen method will depend on the targeted structure and the NMR experimental information available.[29,30]

Considering that the structure of **8** is quite rigid and few significative $^3J_{HH}$ values were available, we chose the DP4+ protocol to elucidate its relative configuration. First, a conformational search of all 32 possible candidate isomers was undertaken. Then, following the DP4+ method, geometric optimization was done at the B3LYP/6-31G* level of theory followed by NMR calculations using PCM/mPW1PW91/6-31+G** (Figure 2). Next, averaged shielding constants were accomplished by calculating Boltzmann populations for each conformation. Finally, experimental and calculated data were compared, obtaining the best match for isomer 1*S**, 2*R**, 3*S**, 4*S**, 7*R**, 8*S**. Importantly, this result was compatible with those assigned in the literature for the related compounds trichocladinols A and B.[3] Moreover, the obtained linear correlation coefficients (R^2's) for carbon and proton are 0.990 and 0.999, respectively, and the CMAE (corrected mean average error) values are 1.3 and 0.1 ppm for carbon and proton, respectively.

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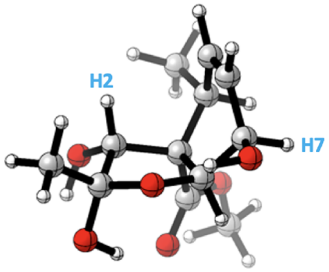

Figure 2. Lowest energy conformations were found for compound **8** at the PCM/mPW1PW91/6-31+G**//B3LYP/6-31G* level of theory.

Compound **9** is a colorless oil whose molecular formula was established as $C_{11}H_{20}O_3$ (two degrees of unsaturation) based on HRESIMS and NMR data. The analysis of ^{1}H, ^{13}C NMR data (Table 2) and the gHSQC experiment revealed the

Table 2. NMR Data of Compound 9 in $CDCl_3$ at 400 (^{1}H) and 100 (^{13}C) MHz

no.	δ_H (J in Hz)	δ_C, type	gHMBC	gCOSY
1		172.9, C		
2		125.9, C		
3	6.63, (dd, 1.5, 10.3)	150.6, CH	C-1, 2, 4, 5, 9, 10	4, 9
4	2.65, m	31.2, CH	C-2, 3, 5, 10	3, 5b, 10
5a	1.19, m	44.6, CH_2	C-3, 4, 6, 7, 10, 11	5b, 6
5b	1.35[a]		C-3, 4, 6, 7, 10, 11	4, 5a, 6
6	1.48[a]	27.8, CH	C-5, 8	5a, 7a, 11
7a	1.36[a]	40.5, CH_2	C-5, 6, 8, 11	6, 7b, 8
7b	1.53[a]		C-5, 6, 8, 11	6, 7a, 8
8	3.67, m	61.1, CH_2	C-6, 7	7a, 7b
9	1.85, (d, 1.5)	12.4, CH_3	C-1, 2, 3	3
10	1.00, (d, 6.6)	20.6, CH_3	C-3, 4, 5	4
11	0.87, (d, 6.4)	19.6, CH_3	C-5, 6, 7	6

[a]Overlapped signals.

presence of two CH_3CH− moieties, a singlet methyl group, two multiplet methylene groups, an oxygenated methylene, a carboxylic acid group, and a trisubstituted olefin. The observed data already accounted for the two degrees of unsaturation, which revealed that **9** was an acyclic compound.

Analysis of the gCOSY results allowed the assignment of an isolated spin system corresponding to C3−C8/C9/C10/C11, where H-3 and H_3-9 showed an allylic coupling constant (J_{3-9} = 1.5 Hz). These data together with the observed correlations in gHMBC experiments of H-3 and H_3-9 to the carboxylic acid confirmed the structure of compound **9** as shown in Figure 1. To define the relative configuration of compound **9**, a conformational search of the two possible diastereoisomers was performed. Again, because of the absence of useful $^3J_{HH}$ values and the relatively small number of candidate structures (205 conformers), the DP4+ method was used following the previously described protocol. The 4*R**, 6*S** relative configuration of **9** was selected with a probability higher than 99%; R^2 of 0.998 and 0.997 for carbon and proton NMR data, respectively; and CMAE values of 1.71 ppm (^{13}C) and 0.07 ppm (^{1}H) for the selected stereoisomer. This proposal was supported by a NOESY correlation between H4−H9 and H4−H11 (Figure 3).

The fungus *Pleotrichocladium opacum* was cultured on a solid rice medium, yielding three additional secondary metabolites

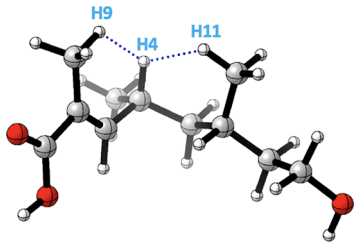

Figure 3. Lowest energy conformations found for compound **9** at the PCM/mPW1PW91/6-31+G**//B3LYP/6-31G* level of theory. Dashed lines represent key NOESY correlations.

(**10**−**12**), in addition to most of the compounds isolated from the culture undertaken using standard conditions previously described. Compounds **10** and **11** were identified as the already known massarilactone D[9] and trichocladinol C,[3] respectively, by comparison with the data described in the literature. Compound **12** was found to have a novel structure (Figure 4).

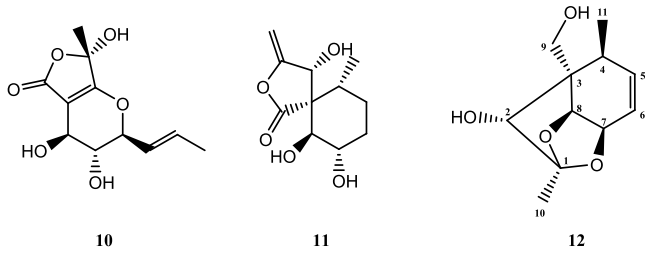

Figure 4. Secondary metabolites isolated from *Pleotrichocladium opacum* (solid rice medium culture).

Compound **12** was obtained as colorless crystals, and its molecular formula $C_{11}H_{16}O_4$ (four degrees of unsaturation) was determined by HRESIMS and NMR data (Table 3). 1D and 2D NMR spectra were very similar to those of **8**, except that the methyl ester in **8** was replaced by an oxygenated methylene (δ_H 3.67 and 3.76 and δ_C 59.8) in **12**. However, the required four degrees of unsaturation for this compound suggested a tricyclic structure. Acetylation of **12** with acetic

Table 3. NMR Data of Compound 12 in MeOH-d_4 at 400 (^{1}H) and 100 (^{13}C) MHz

no.	δ_H (J in Hz)	δ_C, type	gHMBC	gCOSY
1		111.3, C		
2	3.68, s	78.8, CH	C-1, 8, 9	
3		54.1, C		
4	2.73, (ddq, 7.4, 2.7, 2.4)	30.9, CH	C-2, 3, 5, 6, 9, 11	5, 6, 11
5	5.73, (dd, 2.4, 10.0)	138.2, CH	C-3, 4, 7, 11	4, 6
6	5.84, (ddd, 2.7, 5.2, 10.0)	124.1, CH	C-4, 7, 8	4, 5, 7
7	4.00, (dd, 5.2, 3.8)	70.5, CH	C-3, 5, 8	6, 8
8	4.40, (d, 3.8)	77.0, CH	C-1, 2, 7, 9	7
9a	3.67, (d, 11.8)	59.8, CH_2	C-2, 4, 8	9b
9b	3.76, (d, 11.8)		C- 2, 4, 8	9a
10	1.43, s	14.6, CH_3	C-1, 2	
11	1.14, (d, 7.4)	16.2, CH_3	C-3, 4, 5	4
2-OH[a]	4.94, (d, 6.0)		C-1, 2, 3	2
9-OH[a]	4.52, (t, 5.3)		C-3, 9	9a, 9b

[a]Data extracted from DMSO-d_6 measurement.

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anhydride and pyridine afforded diacetylated compound **13**, in agreement with the fact that ^{1}H NMR of compound **12** only showed two exchangeable hydrogens (2-OH and 9-OH). All of this information, besides the molecular weight of compound **12**, led to the conclusion that C-1 must be linked through an ether bridge to C-7, establishing a cyclic acetal. This suggested that **12** was a tricyclic molecule with fused cyclohexene, tetrahydrofuran, and dioxolane rings. The structure of **12** was subsequently confirmed by comparison with the data described in the literature for trichocladinols A and B, whose skeleton is identical to the proposed structure of **12**.

Despite the presence of six stereocenters within this compound, the fact that its backbone is formed by three fused five- and six-membered rings restricts the viable geometric isomers to only four due to structural constraints at C-1, C-3, C-7, and C-8. The *J*-DP4 approach was not used because only one experimentally measured $^{3}J_{HH}$ (H7−H8) was available and, unfortunately, this coupling failed to differentiate between the candidate isomers, as all identified conformations displayed H7−H8 dihedral angles of approximately 40 or 325°, consistent with the experimental coupling constant value (J_{7-8} = 3.8 Hz). Therefore, the DP4+ computational approach was used, providing inconclusive results. Isomers 1*S**, 2*S**, 3*R**, 4*S**, 7*S**, 8*R** and 1*R**, 2*R**, 3*S**, 4*S**, 7*R**, 8*S** gave overall DP4+ probabilities of 56 and 43%, respectively (Table S19a). It has to be noted that experimental measurements were done using CD_3OD and that the use of solvents other than $CDCl_3$ could give more unpredictable results in the NMR calculations, especially for ^{1}H nuclei in polar solvents.[31,32] Doubts arose regarding the previous outcome because the second candidate structure showed the best ^{13}C-DP4+ probability (99%). Moreover, the 1*S**, 2*S**, 3*R**, 4*S**, 7*S**, 8*R** candidate is not compatible with the observation of a NOE correlation between H2 and H11. In consequence, isomers 1*S**, 2*S**, 3*R**, 4*S**, 7*S**, 8*R** and 1*S**, 2*R**, 3*R**, 4*S**, 7*S**, 8*R** were removed from the calculations, and a DP4+ probability of 98% for isomer 1*R**, 2*R**, 3*S**, 4*S**, 7*R**, 8*S** was then obtained. This structure is consistent with the trichocladinol A structure. Furthermore, the observation of a NOE correlation between H2−H11, H4−H8, and H7−H8 (Figure 5), along with the absence of the expected H2−H9 correlation for the C2*S** epimer, suggests that the true structure of compound **12** should possess the 1*R**, 2*R**, 3*S**, 4*S**, 7*R**, 8*S** configuration. The correlation coefficients were 0.994 and 0.981 for carbon and proton, respectively, and the CMAE values were 2.45 and 0.15, respectively. Compound **12** exhibited an optical rotation ($[\alpha]^{20}_{D}$ = +66.8°) similar in sign and magnitude to that of trichocladinol A ($[\alpha]^{20}_{D}$ = +78°), indicating a shared absolute configuration between them.

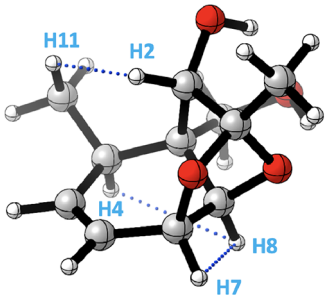


Figure 5. Lowest energy conformations found for compound **12** at the PCM/mPW1PW91/6-31+G**//B3LYP/6-31G* level of theory. Dashed lines represent key NOESY correlations.

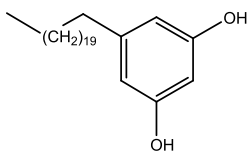
Following the OSMAC approach, the solid culture medium was changed from rice to wheat. Analysis of the fungal culture led to the isolation of a resorcinol derivative, 5-heneicosylresorcinol (**14**),[33] and a new fatty acid (**15**) not previously described in the literature.

Compound **15** was isolated as a yellowish oil with the molecular formula $C_{11}H_{20}O_3$ (two degrees of unsaturation), as deduced from HRESIMS and NMR data. The NMR data of **15** (Table 4) were very similar to those of **9**, and the fact that both compounds had the same molecular formula revealed that **15** was a position isomer of **9.**

Table 4. NMR Data of Compound 15 in $CDCl_3$ at 400 (^{1}H) and 100 (^{13}C) MHz

no.	δ_H (*J* in Hz)	δ_C, type	gHMBC	gCOSY
1		173.1, C		
2		124.5, C		
3	6.78, (d, 10.2)	151.1, CH	C-1, 2, 4, 5, 9, 10	4, 9
4	2.75, m	29.5, CH	C-10	3, 5, 10
5	1.59, m	48.2, CH_2	C-3, 4, 6, 7, 10, 11	4
6		73.1, C		
7	1.45, (q, 7.6)	35.1, CH_2	C-5, 6, 8, 11	8
8	0.88, (t, 7.6)	8.3, CH_3	C-6, 7	7
9	1.86, s	12.0, CH_3	C-1, 2, 3	3
10	1.02, (d, 6.9)	21.6, CH_3	C-3, 4, 5	4
11	1.12, s	26.4, CH_3	C-5, 6, 7	

Comparison of the ^{1}H and ^{13}C NMR data between these compounds revealed the presence of a singlet methyl group bound to an oxygenated quaternary carbon (δ_C 73.1) and an additional triplet methyl group (CH_3−CH_2 moiety) instead of the oxygenated methylene in **9.** gHMBC correlations of H_2-5, H_2-7, H_3-8, and H_3-11 to the oxygenated quaternary carbon suggested its location at C-6, establishing the structure of compound **15** as shown in Figure 6. To elucidate the relative configuration of **15**, a conformational search of the two possible diastereoisomers was performed, obtaining 161 conformers within an energy cutoff of 12 kJ/mol. The complete assignment of compounds **9** and **15** is not trivial, as their acyclic structure allows great conformational flexibility. The obtained results after calculation of the DP4+ probability do not give the expected overwhelming results. With this in mind, the tentative assignment of the relative configuration of compound **15** is 4*R**, 6*R**, which showed the best match between the calculated and experimental data. Excellent correlations were obtained for ^{13}C and ^{1}H (0.998 and 0.999, respectively), and the obtained CMAE values for carbon (1.73 ppm) and proton (0.03 ppm) were also good. Moreover, the



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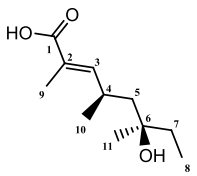

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Figure 6. Secondary metabolites isolated from *Pleotrichocladium opacum* (wheat solid medium culture).

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observed NOEs H4−H9 and H4−H11 reinforce the proposed structure for **15** (Figure 7).

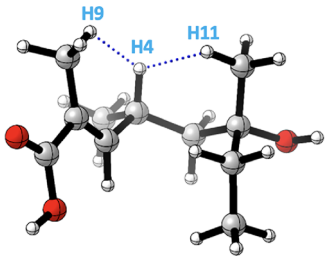

Figure 7. Lowest energy conformations found for compound **15** using the MMFF force field. Dashed lines represent key NOESY correlations.

The addition of epigenetic modifiers to the culture medium was also studied. These molecules are capable of triggering a new metabolic profile, producing new metabolites or increasing their yield.[7]

In this regard, continuing with the OSMAC approach, new fermentations were carried out, choosing solid rice as the culture medium and adding some chemical elicitors. Addition of 5-azacytidine led to the isolation of three new secondary metabolites not previously described in the literature (**16**−**18**), whereas addition of *N*-acetyl-D-glucosamine triggered the production of the known massarilactone G (**19**)[34] and compound **20**, a diastereoisomer of trichocladinol C (**11**).

Compound **16** was isolated and purified to obtain a colorless oil. Analysis by HRESIMS and NMR data (Table 5) determined its molecular formula as $C_{11}H_{16}O_6$ (four degrees of unsaturation). An initial study of the 1D NMR spectra of **16** revealed clear structural similarities with massarilactone C (**1**) isolated from fermentation under standard conditions, but instead of the methyl ester (δ_H 3.73, δ_C 52.2) present in massarilactone C, compound **16** showed a free carboxylic acid group. Further 2D NMR analysis confirmed that the rest of the molecule was identical to massarilactone C. Moreover, computational analysis using the mix-*J*-DP4 method allowed the assignment of the relative configuration of compound **16** as

Table 5. NMR Data of Compound 16 in $CDCl_3$ at 400 (^{1}H) and 100 (^{13}C) MHz

no.	δ_H (*J* in Hz)	δ_C, type	gHMBC	gCOSY
1		171.0, C		
2		64.6, C		
3	4.13, (dd, 5.6)	73.7, CH	C-1, 4, 7, 9	4, 3-OH
4	4.19, m	66.5, CH	C-2, 3, 5, 6	3, 4-OH, 5a
5a	1.85, (ddd, 1.3, 5.6, 15.7)	38.3, CH_2	C-4, 7	4, 5b
5b	2.25, (dd, 15.7, 3.8)		C-3, 4, 6, 7	5a, 6
6	4.23, (dd, 3.8, 1.3)	81.1, CH	C-2, 4, 8, 9	5b
7	1.90, (q, 7.0)	45.8, CH	C-2, 3, 5, 6, 8, 9	8
8	1.06, (d, 7.0)	15.2, CH_3	C-2, 6, 7	7
9	5.10, s	81.6, CH	C-1, 2, 3, 6, 7, 10	
10		206.9, C		
11	2.20, s	28.8, CH_3	C-9, 10	
3-OH	4.04, (d, 5.6)		C-3, 4	3
4-OH	3.10, (d, 3.5)		C-3, 4, 5	4

2*S**, 3*R**, 4*S**, 6*R**, 7*S**, and 9*S**. Briefly, a mixed torsional/low mode conformational sampling protocol was undertaken in the gas phase using the MMFF force field and reoptimizing the conformers with AMBER and MM3 force field. The geometric criteria used to eliminate duplicate structures were MAD 0.5 and an energy cutoff of 12 kJ/mol, NMR calculations were performed at the *J*-DP4 recommended level (B3LYP/6-31G**), and $^3J_{HH}$ used only the Fermi Contact term contribution. In that way, the overall obtain probability is above 90%, a result also confirmed by DP4+ probability (>99%). The R^2 values, obtained with each force field at the mix-*J*-DP4 protocol, are the following: AMBER, 0.997 and 0.973; MM3, 0.997 and 0.958; and MMFF, 0.998 and 0.976 for carbon and proton, respectively, and ^{13}C CMAE values for each force field as follows: AMBER, 2 ppm; MM3, 2.1 ppm; and MMFF, 1.9 ppm. Finally, the obtained ^{1}H CMAE value was 0.2 ppm (for all force fields).

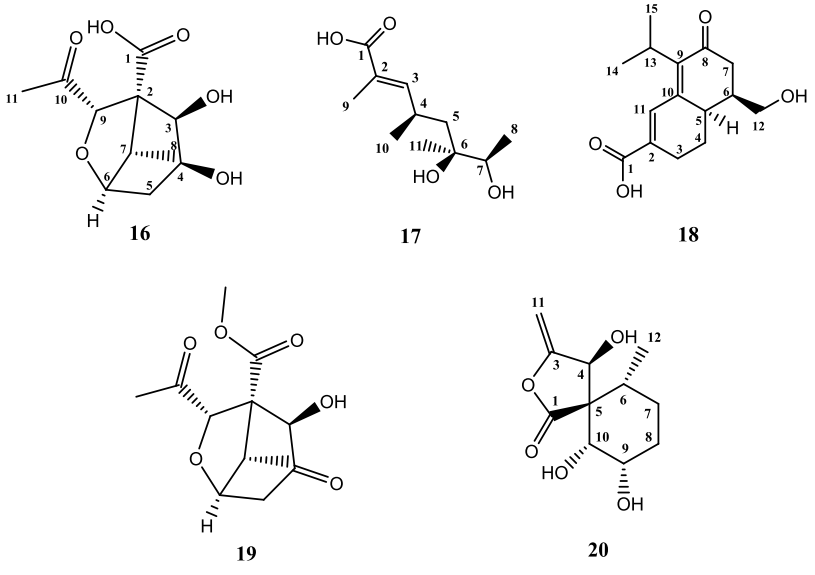

Figure 8. Secondary metabolites isolated from *Pleotrichocladium opacum* (solid rice medium culture and chemical elicitors).

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The observed NOEs between 3OH−4OH, H3−H7, and H6−H8 reinforce the structural proposal for compound **16** (Figure 9).

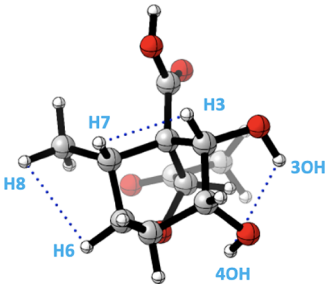

Figure 9. Lowest energy conformations found for compound **16** at the PCM/mPW1PW91/6-31+G**//B3LYP/6-31G* level of theory. Dashed lines represent key NOESY correlations.

This result is clearly in agreement with the stereochemistry of structure **1**, so based on the likewise positive optical rotation for massarilactone C, we tentatively assigned the same absolute configuration to **16** as shown in Figure 8.

Compound **17** (colorless oil) had the molecular formula $C_{11}H_{20}O_4$ (two degrees of unsaturation) based on HRESIMS and NMR data. NMR data (Table 6) were very similar to

Table 6. NMR Data of Compound 17 in $CDCl_3$ at 400 (^{1}H) and 100 (^{13}C) MHz

no.	δ_H (J in Hz)	δ_C, type	gHMBC	gCOSY
1		172.4, C		
2		124.6, C		
3	6.82, (d, 10.2)	151.3, CH	C-1, 2, 4, 5, 9, 10	4, 9
4	2.81, m	29.4, CH		3, 5, 10
5a	1.49, (dd, 14.4, 3.3)	42.3, CH_2	C-3, 4, 10	4, 5b
5b	1.76, (dd, 14.4, 10.0)		C-3, 4, 6, 11	4, 5a
6		75.2, C		
7	3.58, (q, 6.3)	74.8, CH	C-6	8
8	1.14, (d, 6.3)	17.4, CH_3	C-6, 7	7
9	1.89, (d, 1.1)	12.2, CH_3	C-1, 2, 3	3
10	1.05, (d, 6.8)	21.6, CH_3	C-3, 4, 5	4
11	1.15, s	23.7, CH_3	C-5, 6, 7	

those of **15.** The presence of a new methine group (δ_H 3.58, δ_C 74.8) instead of methylene at C-7 suggested the presence of a hydroxy group at this position. The doublet methyl group at C-7, together with the additional oxygen present in the molecular formula of compound **17**, confirmed this suggestion. The remaining substructure was identical to that of **15**, as confirmed after a detailed analysis of the 2D NMR spectra of **17**.

The DP4+ computational approach was used to assign the relative configuration of compound **17**, showing the best probability (>99%) for the isomer 4*R**, 6*S**, 7*R**, which is in agreement with their structural analogs **9** and **15**. This compound showed 187 conformations even though it has four isomers, and the structure is similar to that of **9** and **15**. This could be due to the plausible formation of a hydrogen bond between 6-OH and 7-OH. Geometric optimization was done at the B3LYP/6-31G* level of theory followed by NMR calculations using PCM/mPW1PW91/6-31+G** (Figure 10)

The third new natural product produced by *Pleotrichocladium opacum* using 5-azacytidine as an epigenetic modifier on

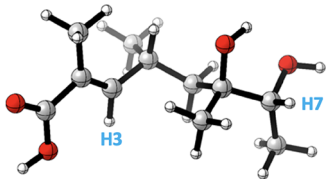

Figure 10. Lowest energy conformations found for compound **17** at the PCM/mPW1PW91/6-31+G**//B3LYP/6-31G* level of theory.

the solid rice medium was a colorless amorphous solid (**18**) with the molecular formula $C_{15}H_{20}O_4$ determined based on HRESIMS and NMR data (six degrees of unsaturation). The ^{1}H and ^{13}C NMR spectra in conjunction with gCOSY and gHSQC experiments revealed the presence of two doublet methyl groups and a septuplet methine forming an isopropyl moiety; four methylene groups, one of them oxygenated; two additional methine groups; a trisubstituted olefin; three olefinic quaternary carbons (δ_C 137.1, 142.8, and 146.0); and two carbonyl groups, a ketone (δ_C 199.9) and a carboxylic acid (δ_C 169.1). That meant four out of the six degrees of unsaturation required by the molecular formula, which suggested that the molecule should be bicyclic. gCOSY experiments allowed the assignment of two isolated spin systems corresponding to C3−C7/C12 and C13/C14/C15.

Comprehensive analysis of all correlations observed in gHMBC (Table 7) concluded that the structure of **18** consisted of a cyclohexene−cyclohexenone fused scaffold. gHMBC correlations of H-13 to C-8, C-9, and C-10 anchored the isopropyl group to C-9. H-11 and H_2-3 correlation to the carboxylic acid carbon at δ_C 169.1 ppm placed that group bounded at C-2. Finally, H_2-12 correlations to C-5, C-6, and

Table 7. NMR Data of Compound 18 in $CDCl_3$ + MeOH-d_4 at 400 (^{1}H) and 100 (^{13}C) MHz

no.	δ_H (J in Hz)	δ_C, type	gHMBC	gCOSY
1		169.1, C		
2		137.1, C		
3a	2.30, m[a]	25.5, CH_2	C-1, 2, 5, 11	3b, 4a, 4b
3b	2.68, m[a]		C-1, 2, 5, 11	3a, 4a, 4b
4a	1.60, (qd, 13.0,4.7)	26.8, CH_2	C-3, 5, 10	3a, 3b, 4b, 5
4b	1.85, m			3a, 3b, 4a, 5
5	2.72, m[a]	38.5, CH	C-4, 6, 9, 12	4a, 4b, 6
6	2.30, m[a]	39.8, CH		5, 7a, 7b, 12a, 12b
7a	2.48, (dd, 16.3,5.1)	42.2, CH_2	C-5, 8, 12	6, 7b
7b	2.75, m[a]		C-5, 8, 12	6, 7a
8		199.9, C		
9		142.8, C		
10		146.0, C		
11	7.72, (d, 2.5)	133.1, CH	C-1, 2, 3, 5, 9, 10	
12a	3.46, (dd, 4.8,11.1)	60.0, CH_2	C-5, 6, 7	6, 12b
12b	3.69, (dd, 9.0,11.1)		C-5, 6, 7	6, 12a
13	3.17, (sp, 7.1)	26.8, CH	C-8, 9, 10, 14, 15	14, 15
14	1.15, (d, 7.1)	20.9, CH_3	C-9, 13, 15	13
15	1.18, (d, 7.1)	21.3, CH_3	C-9, 13, 14	13

[a]Overlapped signals.

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C-7 confirmed the presence of the H_2-12 methylene linked to the C-6 position. Additional gHMBC correlations supported the proposed novel structure of compound **18** as shown in Figure 8.

The mix-*J*-DP4 computational analysis was performed by following the same workflow previously described. $^3J_{H12aH6}$ and $^3J_{H12bH6}$ showed experimental values of 4.8 and 9 Hz. In that sense, the i*J*-DP4 procedure was achieved by removing conformers with two small values for $^3J_{H12aH6}$ and $^3J_{H12bH6}$. The obtained result gives the best probability, over 99%, for isomers 5*R** and 6*R**. R^2 values for carbon get values from 0.988 for AMBER to 0.999 and 0.998 for MM3 and MMFF; on the other side for proton, the values are more homogeneous at 0.988−0.989 in all cases. CMAE values for the right isomer using AMBER are 4.8 ppm for ^{13}C and 0.1 ppm for ^{1}H; MM3 gives lower values of 1.9 ppm for carbon and 0.2 for proton; finally, MMFF gives values of 2.2 ppm for carbon and 0.1 ppm for proton, which led to the assignment of the relative configuration for compound **18**. Geometric optimization was done at the B3LYP/6-31G* level of theory followed by NMR calculations using PCM/mPW1PW91/6-31+G** (Figure 11).

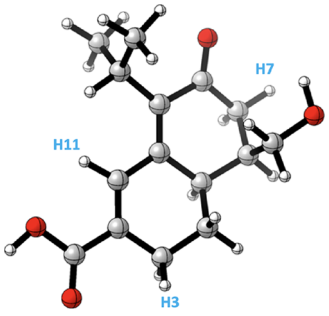

Figure 11. Lowest energy conformations found for compound **18** at the PCM/mPW1PW91/6-31+G**//B3LYP/6-31G* level of theory.

Compound **20** at first sight seemed to be trichocladinol C due to the observable correlations in the 2D NMR spectra; however, the ^{1}H NMR spectrum showed significant differences in some chemical shifts and coupling constants (Table 8). This suggested that compound **20** was a diastereoisomer of trichocladinol C. DP4+ analysis of **20** was performed and confirmed a relative configuration of 4*S**, 5*S**, 6*R**, 9*S**, and 10*R** for the compound. Moreover, the $^3J_{H9H10}$ (2.4 Hz) agrees with the computed structures for the most probable isomer. The linear correlation coefficient values for the most probable isomer are 0.999 and 0.996 for ^{13}C and ^{1}H, respectively, and the obtained CMAE values were 1.1 ppm (^{13}C) and 0.1 ppm (^{1}H). The observed NOEs between H12−H4 and H6−H9−H7b reinforce the proposed structure for compound **20** (Figure 12).

Massarilactones, massarigenins, and the closely related trichocladinols may share the same biosynthetic pathway. Biogenetically, by comparison of the biogenesis of arthropsolide A,[35] all of them could be derived from the condensation of a tetraketide precursor and a malic acid unit followed by a series of enzymatic reactions.

A possible biosynthetic pathway is proposed in Figure 13.

Finally, an exhaustive study on the effect of microbial interactions on the production of new secondary metabolites with different biological activities was conducted by coculturing the fungus *Pleotrichocladium opacum* with *Echinocatena* sp.

Table 8. NMR Data of Compound 20 in MeOH-d_4 at 400 (^{1}H) and 100 (^{13}C) MHz

no.	δ_H (*J* in Hz)	δ_C, type	gHMBC	gCOSY
1		178.5, C		
3		159.7, C		
4	5.42, (t, 2.5)	69.4, CH	C-3, 5, 6, 10, 11	11a, 11b
5		57.1, C		
6	1.75, (ddq, 4.3, 7.0, 13.7)	40.4, CH	C-1, 4, 5, 7, 8, 10, 12	7a, 7b, 12
7a	1.31, (dtd, 2.3, 4.8, 13.7)	26.0, CH_2	C-5, 6, 8, 9, 12	6, 7b, 8a, 8b
7b	1.97, (qd, 4.3, 13.7)		C-5, 6, 8, 9, 12	6, 7a, 8a, 8b
8a	1.61, (tdd, 2.4, 4.8, 13.7)	31.7, CH_2	C-6, 7	7a, 7b, 8b, 9
8b	1.87, (dtd, 2.3, 3.9, 13.7)		C-6, 9, 10	7a, 7b, 8a, 9
9	4.02, (dt, 2.4, 3.9)	71.2, CH	C-5, 7, 10	8a, 8b, 10
10	3.83, (d, 2.4)	72.9, CH	C-1, 4, 5, 6, 8, 9	9
11a	4.57, (t, 2.5)	87.0, CH_2	C-3, 4	4, 11b
11b	4.70, (t, 2.5)		C-3, 4	4, 11a
12	1.08, (d, 7.0)	17.1, CH_3	C-5, 6, 7	6

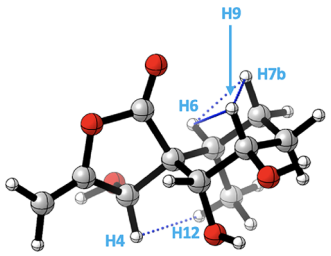

Figure 12. Lowest energy conformations found for compound **20** using the MMFF force field. Dashed lines represent key NOESY correlations.

in a solid PDA medium. As a result, five further anthraquinone and benzophenone derivatives (**21**−**25**) were isolated (Figure 14). The anthraquinone chemical class is usually reported with antibiotic activity, implying a possible defense reaction of the producer fungus in the coculture. Compound **21** proved to be a new natural anthraquinone previously unreported in the literature.

Known compounds **22**−**25** were identified as carviolin (**22**),[36] emodin 6,8-dimethyl ether (**23**),[37] questin (**24**),[38] and sulochrin (**25**)[39] after extensive spectroscopic analysis and comparison with data reported in the literature.

Compound **21** was a yellow, amorphous solid. HRESIMS gave a molecular ion at *m*/*z* of 341.0137 ([M + Na]$^+$) and an isotopic peak at *m*/*z* 343.0108 with a ratio of 3:1, suggesting a molecular formula of $C_{16}H_{11}ClO_5$ (11 degrees of unsaturation). Comparison of the ^{1}H and ^{13}C NMR data (Table 9) with those of questin showed the similarity between these two molecules, the only difference being the removal of the aromatic hydrogen H-7 by a chlorine atom. gHMBC correlation of H-5 to the new quaternary aromatic carbon C-7 (δ_C 122.7 in DMSO-d_6) clearly established the chlorine atom position.

In conclusion, an axenic fermentation of *Pleotrichocladium opacum* on the JSA medium afforded nine natural products (**1**−**9**) including two new compounds (**8** and **9**). A switch from the liquid JSA medium to solid rice yielded three

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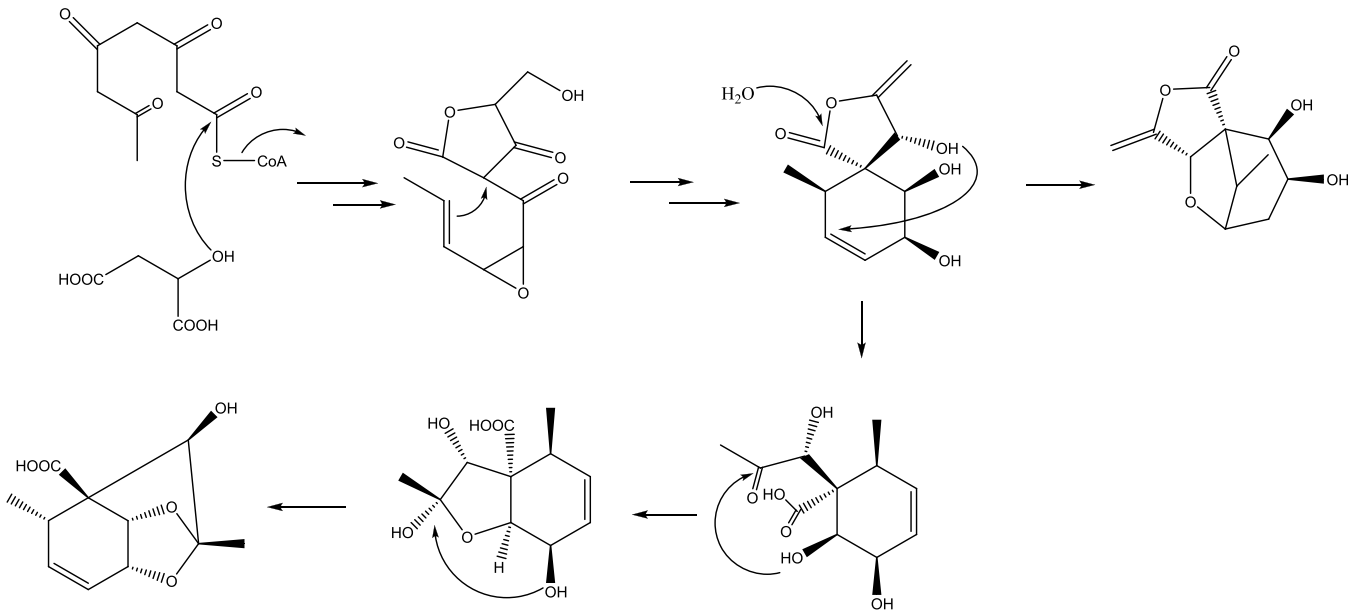

Figure 13. Possible metabolic pathway of massarilactones, massarigenins, and trichocladinols.

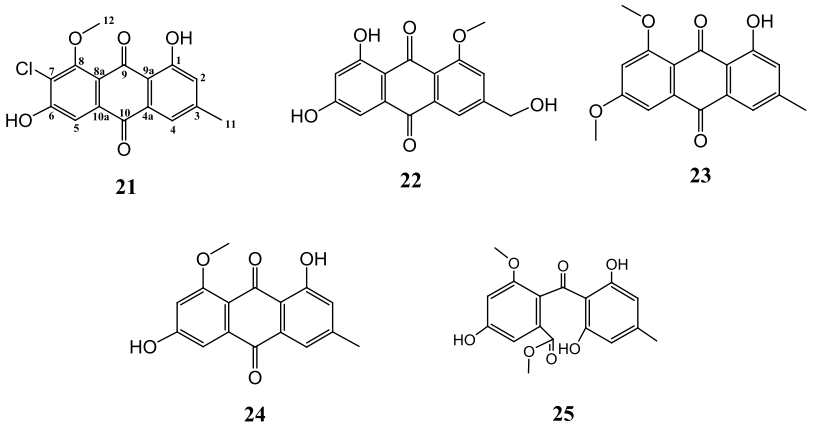

Figure 14. Secondary metabolites isolated from cocultivation of *Pleotrichocladium opacum* with *Echinocatena* sp.

additional compounds (**10**−**12**), with compound **12** being a novel trichocladinol. Changing from solid rice to wheat resulted in the production of two further secondary metabolites (**14** and **15**), where **15** was a novel short-chain fatty acid. Addition of 5-azacytidine to the rice solid medium caused the accumulation of three additional novel natural products (**16**−**18**), i.e., a novel massarilactone, a new short-chain fatty acid, and a novel sesquiterpenoid, respectively. Addition of *N*-acetyl-D-glucosamine to the rice solid medium triggered the production of two additional compounds (**19** and **20**), where compound **20** is a novel diastereoisomer of trichocladinol C. Finally, cocultivation of the fungus *Pleotrichocladium opacum* with *Echinocatena* sp. in a solid PDA medium led to the production of five additional natural products (**21**−**25**), with compound **21** being a novel anthraquinone derivative.

All compounds were subjected to a cytotoxicity assay against the tumor cell lines A549, H116, PSN1, and T98G (Table 10). Massarilactone C (**1**) showed weak cytotoxicity against PSN1 with an IC_{50} of 19.3 μM. Massarilactone H (**4**) exhibited weak cytotoxicity against A549, H116, PSN1, and T98G with an IC_{50} value of 22.3 μM. Massarigenin A (**7**) was active against H116 and PSN1 with a value of IC_{50} of 22.1 μM. Emodin 6,8-dimethyl ether (**23**) showed a similar cytotoxicity against A549, H116, and PSN1 with an IC_{50} of 16.7 μM. Sulochrin (**25**) exhibited cytotoxic activity against A549, H116, PSN1, and T98G with IC_{50} values of 3, 3, 3, and 30 μM, respectively, and antiangiogenesis activity against BAEC cells with an IC_{50} value of 3 μM. The remaining compounds were inactive against the cell lines tested.

The antimicrobial activity of all compounds was also tested against *B. subtilis*, *S. aureus*, and *K. rhizophila* (Table 10). Massarilactone H (**4**) showed weak antibacterial activity against *K. rhizophila* with an MIC value of 71.3 μM. The new compound **21** exhibited weak activities against *B. subtilis*, *S. aureus*, and *K. rhizophila*, with MIC values of 200, 50, and 25 μM, respectively. The remaining compounds were inactive against the tested bacteria.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-2000 polarimeter using MeOH as solvent. ^{1}H and ^{13}C NMR data were obtained on a Varian "Mercury 400" spectrometer at 400 and 100 MHz,

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Table 9. NMR Data of Compound 21 in DMSO-d_6 at 400 (^{1}H) and 100 (^{13}C) MHz

no.	Δ_H (J in Hz)	δ_C, type	gHMBC	gCOSY
1		161.8, C		
2	7.15, s	124.1, CH	C-1, 4, 9a, 11	
3		146.9, C		
4	7.43, s	119.2, CH	C-2, 9a, 10, 11	
4a		132.1, C		
5	7.43, s	111.8, CH	C-7, 8a, 10, 10a	
6		159.4, C[b]		
7		122.7, C[a]		
8		158.8, C		
8a		115.9, C[a]		
9		185.4, C		
9a		114.6, C		
10		182.1, C		
10a		133.9, C		
11	2.39, s	21.4, CH_3	C-2, 3, 4	
12	3.85, s	60.7, CH_3	C-8	
1-OH	13.2, s		C-1, 2, 9a	

[a]Chemical shifts of C-7 and C-8a were assigned through the correlations observed in gHMBC. [b]Data extracted from $CDCl_3$ measurement.

respectively. Chemical shifts are reported in parts per million relative to solvent ($CDCl_3$: δ = 7.26/77.16 ppm; [D_6]DMSO: δ = 2.50/39.52 ppm; [D_4]MeOH: δ = 3.31/49.00 ppm). HPLC-MS was carried out with an Agilent UHPLC 1290 Infinity II coupled to an Agilent TOF 6230. HPLC analysis was performed with a Zorbax Eclipse plus C18 column using a linear gradient from 20 to 100% methanol in 8 min, post time of 2 min, and DAD analysis range of 200−600 nm signal obtained at 220 nm. Mass spectrometer conditions in ESI-Positive were 100 V fragmentor and mass range (m/z) = 100−3200. The analysis was performed with the Masshunter suite software (Agilent Technologies) version B.08.00. An Agilent 1200 series liquid chromatograph with a photodiode-array and evaporative light scattering detectors was used for HPLC analysis and recording UV spectra. Semipreparative HPLC purification was conducted on an Agilent 1100 series (Kinetex 5 μm EVO C18 10 column, 100 × 21.2 mm).

Fungal Material. *HT-GU-SUES-1371.* The microorganism was isolated from a soil sample collected on the coast of Asturias (Spain). The strain was transferred from the collection of Dr. J. Guarro, Universitat Rovira i Virgili, and was deposited at the Spanish Type Culture Collection (CECT) under the number CECT 21234. Taxonomical determination was achieved after sequencing analysis of the ITS1-5.8S-ITS2 region of the rDNA. Comparing the sequence obtained with the sequences deposited at GenBank databases, a 99% match is obtained compared with *Pleotrichocladium opacum*. Colonies show a white cottony mycelial growth that becomes gray after 15 days. They reached 9 cm diameter in 15 days at 25 °C on potato dextrose agar in a culture chamber that maintains a humidity of 42%. The strain sporulates forming one or three clavate type alleurioconidia, brown colored at the tip and paler at the basal cell. A pure culture of HT-GU-SUES-1371 was kept frozen at −70 °C in 20% glycerol.

HL-84-MIS4-AD04. The microorganism was isolated from a gorgonian sample collected in Equatorial Guinea at a depth of 23 m in a reef close to Bioko Island. The strain was deposited at the Spanish Type Culture Collection (CECT) under CECT 21235. Taxonomic determination was achieved after sequencing analysis of the ITS1-5.8S-ITS2 region of the rDNA. Comparing the sequence obtained with the sequences deposited in the GenBank databases, a 94% match is obtained with a *Fusicladium* sp. isolated from the marine sponge *Amphimedon viridis* (GenBank JN837045; identities 795/846 (94%), five gaps). The second match is with *Echinocatena arthrinioides* (GenBank MH107890.1; identities 438/478 (92%), five gaps). The third and fourth matches are with *Echinocatena arthrinioides* and *Sympoventuriaceae* sp. All these species belong to different genera of the family Sympoventuriaceae. The low match value could be interpreted as meaning that this strain could correspond to a new genus and species belonging to Sympoventuriaceae. In the morphological evaluation of the strain, colonies show a yellowish green cottony mycelium with light brown exudates. They produce a brown pigment that diffuses on agar. Growth is restricted, reaching 5 cm in diameter in 30 days at 25 °C on potato dextrose agar with sea salts in a culture chamber maintained at 42% humidity. Conidiogenesis is easily obtained by the microslide culture technique on PDA agar. Conidiogenous cells appear as single chains that develop into branched acropetal chains separated by thick septa. Conidia are spherical, brown, and aseptate. The morphological description fits more closely to species belonging to the second and third match *Echinocatena arthrinioides* than to the first match *Fusicladium* sp., so the taxonomic assignment is doubtful but remains within the family Sympoventuriaceae. A pure culture of HL-84-MIS4-AD04 was kept frozen at −70 °C in 20% glycerol.

Fermentation and Cocultivation. Fermentation of the fungus was conducted in eight 40 mL Erlenmeyer flasks with 10 mL of JSA seeding medium each consisting of 2% oatmeal, 2% malt extract, 0.01% KH_2PO_4, and 0.005% $MgSO_4{\cdot}7H_2O$. Two 1 cm diameter chopped plugs of a well-developed fungal culture on PDA were used to inoculate each flask. The flasks were grown at 25 °C on a rotary shaker at 200 rpm in the dark

Table 10. Biological Activities of Compounds 1−25[a]

	antimicrobial[b]			cytotoxic[c]				antiangiogenic[c]
compound	B. subtilis	S. aureus	K. rhizophila	A549	H116	PSN1	T98G	BAEC
1	>200	>200	>200	>30	>30	19.3	>30	>30
4	>200	>200	71.3	22.3	22.3	22.3	22.3	>30
7	>200	>200	>200	>30	22.1	22.1	>30	>30
21	200	50	25	>30	>30	>30	>30	>30
23	>200	>200	>200	16.7	16.7	16.7	>30	>30
25	>200	>200	>200	3	3	3	30	3

[a]Other compounds were inactive. [b]MIC values in μM. [c]IC_{50} values in μM

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for 48 h and used as a first-stage inoculum. Five milliliters per flask of the first-stage inoculum was used to inoculate eight 250 mL Erlenmeyer flasks each with 40 mL of the same culture medium and incubated under the same conditions. After 48 h of incubation, they were used as second-stage inoculum. The 4 L fermentation was carried out in sixteen 2 L Erlenmeyer flasks with 250 mL of fermentation medium each in the same culture medium and under the same conditions of agitation and temperature described above for 7 days. A volume of 15 mL of the second stage per flask was used as inoculum.

Co-cultivation experiment with *Echinocatena* sp. (CECT 21235) was conducted on the PDA solid medium. The strains were two-point inoculated, one on each side, on sixty 90 mm Petri plates containing PDA and incubated for 28 days at 25 °C in the dark.

OSMAC Experiments. In addition to the fermentation under standard conditions, rice and wheat were used as solid medium. Solid-state fermentation in rice was carried out in sixteen 2 L Erlenmeyer flasks with a fermentation medium containing 125 g of parboiled rice and 94 mL of distilled H_2O, whereas a medium containing 19.9% wheat, 0.18% $C_6H_{12}O_6$, 0.9% $C_4H_4Na_2O_6$, 0.045% NH_4NO_3, 1.33 × 10−5% $FeSO_4 \cdot 7H_2O$, 1.33 × 10−5% $MnCl_2 \cdot 4H_2O$, 1.33 × 10−5% $ZnSO_4 \cdot 7H_2O$, and 79.75% distilled H_2O p/p was used for solid-state fermentation in wheat. The rest of the cultivation procedure was the same as described above for cultivation under standard conditions, except that the flasks were incubated at 25 °C in the dark for 28 days. For the feeding experiment using 5-azacytidine and *N*-acetyl-D-glucosamine as epigenetic factors, these were added to the rice medium at a concentration of 162 and 0.5 μM, respectively.

Extraction and Isolation. The fermentation broth (4 L) of *Pleotrichocladium opacum* under standard conditions (liquid medium) was stirred with Amberlite XAD1180N resin (10% v/v), which was previously added. Then, culture broth was filtered through Celite, and the mycelial cake with the resin was extracted with 4 L of a mixture of EtOAc/MeOH (3:1). The resultant suspension was filtered and partitioned between EtOAc and water. The organic layer was dried, and the crude extract (11.25 g) was fractionated by VFC (vacuum flash chromatography) on a silica gel, eluting with a stepwise gradient of hexane/EtOAc/MeOH to give 12 fractions (1F1−1F12). Fraction 1F11 (eluted with EtOAc/MeOH 25:75) was further fractionated by normal-phase liquid chromatography using a stepwise gradient of CH_2Cl_2 and acetone to give **1** (94.8 mg) and **2** (126 mg). Fraction 1F7 (eluted with EtOAc) was also fractionated by normal-phase liquid chromatography using a gradient of CH_2Cl_2/acetone as mobile phase followed by semipreparative HPLC purification with a gradient of MeOH/H_2O (from 20 to 100% MeOH over 21 min, stayed at 20% MeOH for 7 min at a flow rate of 16.61 mL/min) as the eluting system to afford **3** (36 mg) and **4** (8.5 mg). Following a similar procedure as described for fraction 1F7, compounds **5** (6.1 mg), **6** (11.7 mg), and **9** (13.7 mg) were isolated from fraction 1F8−9 (eluted with EtOAc/MeOH 75:25), whereas compounds **7** (87.2 mg) and **8** (28 mg) were obtained from fraction 1F6 (eluted with hexane/EtOAc 2:8).

In the case of solid-state fermentations (OSMAC and cocultivation), the contents of the flasks (or Petri plates) were collected and homogenized with the aid of an electric mixer followed by extraction with 100% v/v of a mixture of EtOAc/MeOH (3:1). The chromatographic workup of the OSMAC and cocultivation extracts followed the same procedure as described for the standard conditions culture. Silica gel vacuum flash chromatography using a stepwise gradient of hexane/EtOAc/MeOH was used for separation of the crude extracts obtained from the rice medium (40.1 g), wheat medium (21.4 g), rice medium with addition of 5-azacytidine (45.8 g), *N*-acetyl-D-glucosamine (67.1 g), and cocultivation (2.23 g). A total of 12 fractions (R1F1−R1F12) were obtained from the rice culture extract. Fraction R1F8−9 (eluted with EtOAc/MeOH 75:25) was further fractionated by normal-phase liquid chromatography using a step gradient of CH_2Cl_2 and acetone to yield 10 subfractions (R2F1−R2F10). Subfraction R2F8−10 (eluted with CH_2Cl_2/acetone 1:1) was purified by reversed-phase liquid chromatography using a step gradient of acetone/H_2O to give **10** (35.2 mg), whereas subfractions R2F7 and R2F6 (eluted with CH_2Cl_2/acetone 65:35) were also purified by acetone/H_2O reversed-phase liquid chromatography, yielding **11** (14.5 mg) and **12** (39.4 mg), respectively. Twelve fractions were obtained from wheat culture (W1F1−W1F12) after VFC. Compound **14** (65.9 mg) was isolated from W1F4−5 (eluted with hexane/EtOAc 3:7) after purification by normal-phase liquid chromatography using a step gradient of hexane/ether. Subfraction W1F6 (eluted with hexane/EtOAc 2:8) was fractionated by normal-phase liquid chromatography, using a gradient of CH_2Cl_2/acetone as mobile phase, followed by semipreparative HPLC purification with a gradient of MeOH/H_2O (from 40% to 100% MeOH over 20 min, stayed at 40% MeOH for 5 min at a flow rate of 16.61 mL/min) as eluting system to afford **15** (6.4 mg). Fraction A1F10 (eluted with EtOAc/MeOH 1:1), obtained by VFC from rice culture after the addition of 5-Azacytidine extract, was fractionated by normal-phase liquid chromatography using a step gradient of CH_2Cl_2/MeOH to yield 12 subfractions (A2F1−A2F12). Compound **16** (27.1 mg) was isolated from subfraction A2F4 (eluted with CH_2Cl_2/MeOH 94:6) after purification by normal-phase liquid chromatography with a gradient of CH_2Cl_2/acetone, whereas compound **17** (1.3 mg) was yielded from subfraction A2F10 (eluted with CH_2Cl_2/MeOH 1:1) after semipreparative HPLC purification using a MeOH/H_2O gradient (from 15 to 70% MeOH over 20 min, stayed at 15% MeOH for 7 min at a flow rate of 21.7 mL/min). Fraction A1F11 (eluted with EtOAc/MeOH 25:75) was also fractionated following the same procedure as that described for compound **17** to give **18** (4.4 mg). Compounds **19** (9.3 mg) and **20** (49.4 mg) were isolated from fraction N1F6−7 (rice culture after addition of *N*-acetyl-D-glucosamine) (eluted with EtOAc) by normal-phase liquid chromatography with a gradient of CH_2Cl_2/acetone followed by reversed-phase liquid chromatography with a gradient of acetone/H_2O. Finally, fraction C1F5−6 (eluted with hexane/EtOAc 2:8), obtained from the coculture extract after VFC, was fractionated by normal-phase liquid chromatography using a hexane/EtOAc gradient (C2F1−C2F10) to afford compounds **21** (4.5 mg), **24** (46.4 mg), and **25** (29.3 mg). Subfractions C2F13 (eluted with EtOAc) and C2F5 (eluted with hexane/EtOAc 1:1) were further purified by reversed-phase liquid chromatography using a MeOH/H_2O gradient to give **22** (3.9 mg) and **23** (7.8 mg), respectively.

Compound ***8***. Colorless solid; UV (MeOH) λ_{max} (end of absorption); ^{1}H and ^{13}C NMR data see Table 1; HRESIMS m/z 281.0994 $[M + Na]^+$ (calcd. for $C_{12}H_{18}O_6Na$, 281.1001).

Compound ***9***. Colorless oil; $[\alpha]^{20}_D$ = +10.4° (c 0.133, MeOH); UV (MeOH) λ_{max} 218 nm; ^{1}H and ^{13}C NMR data

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see Table 2; HRESIMS m/z 223.1321 $[M + Na]^+$ (calcd. for $C_{11}H_{20}O_3Na$, 223.1310).

Compound **12**. Colorless crystals; $[\alpha]^{20}_D$ = +66.8° (c 0.33, MeOH); UV (MeOH) λ_{max} 210 nm (end of absorption); ^1H and ^{13}C NMR data see Table 3; HRESIMS m/z 235.0918 $[M + Na]^+$ (calcd. for $C_{11}H_{16}O_4Na$, 235.0946).

Compound **15**. Yellowish oil; UV (MeOH) λ_{max} 220 nm; ^1H and ^{13}C NMR data see Table 4; HRESIMS m/z 223.1319 $[M + Na]^+$ (calcd. for $C_{11}H_{20}O_3Na$, 223.1310).

Compound **16**. Colorless oil; $[\alpha]^{20}_D$ = +104.2° (c 0.67, MeOH); UV (MeOH) λ_{max} 210, 278 nm; ^1H and ^{13}C NMR data see Table 5; HRESIMS m/z 245.1033 $[M + H]^+$ (calcd. for $C_{11}H_{17}O_6$, 245.1025).

Compound **17**. Colorless oil; UV (MeOH) λ_{max} 222 nm; ^1H and ^{13}C NMR data see Table 6; HRESIMS m/z 239.1266 $[M + Na]^+$ (calcd. for $C_{11}H_{20}O_4Na$, 239.1259).

Compound **18**. Colorless amorphous solid; $[\alpha]^{20}_D$ = +210.2° (c 0.13, MeOH); UV (MeOH) λ_{max} 310 nm; ^1H and ^{13}C NMR data see Table 7; HRESIMS m/z 265.1448 $[M + H]^+$ (calcd. for $C_{15}H_{21}O_4$, 265.1440).

Compound **20**. Colorless oil; $[\alpha]^{20}_D$ = +9.4° (c 1.06, MeOH); UV (MeOH) λ_{max} (end of absorption); ^1H and ^{13}C NMR data see Table 8; HRESIMS m/z 229.1043 $[M + H]^+$ (calcd. for $C_{11}H_{17}O_5$, 229.1076).

Compound **21**. Yellow amorphous solid; UV (MeOH) λ_{max} 220, 276, 416 nm; ^1H and ^{13}C NMR data see Table 9; HRESIMS m/z 341.0195 $[M + Na]^+$ (calcd. for $C_{16}H_{11}ClO_5Na$, 341.0193).

Computational methods. Calculations were performed following the general workflow described for DP4+ and mix-*J*-DP4 methods.[21,23] In that way, all possible isomers were generated, and conformational searches were accomplished employing the mixed torsional/low mode conformational sampling protocol in the gas phase using the MMFF force field and reoptimizing the conformers with the AMBER and MM3 force fields in the mix-*J*-DP4 method. NMR calculations were achieved using the conformations under 12 kJ/mol energy cutoff and MAD 0.5 Å. DFT levels of theory as recommended for DP4+ (PCM/mPW1PW91/6-31+G**//B3LYP/6-31G*) and *J*-DP4 (B3LYP/6-31G**) in Gaussian 16[40] were used to calculate magnetic shielding constants by means of the gauge including the atomic orbital method[41−44] and $^3J_{HH}$ if necessary, employing exclusively the Fermi Contact term contribution. All molecular models figures were performed using CYLview20.[45]

Biological Assays. The antimicrobial activity of the compounds was investigated using the microdilution method. Compounds were dissolved in a mixture of MeOH/DMSO (9:1) and added to the broth. The resulting final DMSO concentration in the assay was 1%. The direct colony suspension method was employed for preparation of the inoculum, and the MIC values for each strain were the lowest concentration of the compound at which no growth could be detected. Control of the noninoculated medium and of inoculated media without treatments was carried out in parallel.

Cell culture cytotoxicity assays were performed as described[46,47] using human lung carcinoma cells A549,[48] colorectal carcinoma cells HCT-116,[49] pancreatic adenocarcinoma cells PNS1,[50] and glioblastoma multiforme cells T98G.[51]

The tube formation inhibition assay was performed as described[52] using bovine aortic endothelial cells (BAECs).[53]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06299.

Computational section and NMR spectra of compounds **8**, **9**, **12**, **15**−**18**, **20**, and **21** (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Antonio Hernández Daranas − *Instituto de Productos Naturales y Agrobiología, Consejo Superior de Investigaciones Científicas (IPNA-CSIC), San Cristobal de La Laguna 38206, Spain;* orcid.org/0000-0001-8376-7941; Email: adaranas@ipna.csic.es

José M. Sánchez López − *Biomar Microbial Technologies, Parque Tecnológico de León, León 24009, Spain;* orcid.org/0009-0000-4060-3306; Email: jm.sanchez@biomarmt.com

Authors

Víctor Rodríguez Martín-Aragón − *Biomar Microbial Technologies, Parque Tecnológico de León, León 24009, Spain;* orcid.org/0000-0002-1056-1384

Mónica Trigal Martínez − *Biomar Microbial Technologies, Parque Tecnológico de León, León 24009, Spain*

Cristina Cuadrado − *Instituto de Productos Naturales y Agrobiología, Consejo Superior de Investigaciones Científicas (IPNA-CSIC), San Cristobal de La Laguna 38206, Spain;* orcid.org/0000-0001-9282-1575

Antonio Fernández Medarde − *Biomar Microbial Technologies, Parque Tecnológico de León, León 24009, Spain*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c06299

Author Contributions

All listed authors contributed to this work. Víctor Rodríguez Martín-Aragón: performed most of the isolation, purification, and structural elucidation of the compounds described and wrote this paper. Mónica Trigal Martínez: fungal fermentation and some biological assays. Cristina Cuadrado and Antonio Hernández Daranas: QM-NMR studies to establish the relative configuration of the new compounds and contribution to the final manuscript. Antonio Fernández: advised, assisted, and shared the tasks of manuscript revision. José M. Sánchez López: project leader organizing and guiding the experiments and manuscript writing.

Notes

The authors declare no competing financial interest.

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