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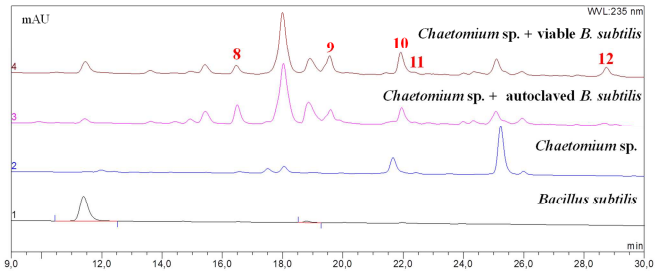

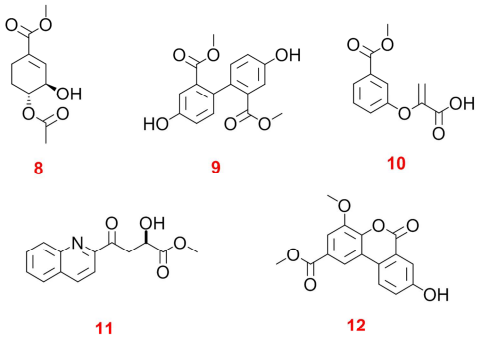

Inducing secondary metabolite production by the endophytic fungus *Chaetomium* sp. through fungal-bacterial co-culture and epigenetic modification

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ABSTRACT

Co-culturing the fungal endophyte *Chaetomium* sp. with the bacterium *Bacillus subtilis* on solid rice medium resulted in an up to 8.3-fold increase in the accumulation of constitutively present metabolites that included a 1:1 mixture of 3- and 4-hydroxybenzoic acid methyl esters (**1** and **2** respectively), and the polyketides acremonisol A (**3**), SB236050 (**4**), and SB238569 (**5**). In addition, seven compounds including isosulochrin (**6**), protocatechuic acid methyl ester (**7**), as well as five new natural products (**8**-**12**) were detected in the co-cultures, but not in axenic fungal cultures. Treatment of *Chaetomium* sp. with the epigenetic modifier suberoylanilide hydroxamic acid or 5-azacytidine resulted in an enhanced accumulation of **6,** which was likewise detected during co-culture. Compound **5** showed strong cytotoxicity against the mouse lymphoma L5178Y cell line with an IC_{50} value of 1 μM, as well as weak antibacterial activity against *B. subtilis* with an MIC value of 53 μM.

Keywords: Co-cultivation; *Chaetomium* sp.; *Bacillus subtilis*; Epigenetic modifiers; natural products

1. Introduction

Microorganisms, including soil-dwelling bacteria and fungi, produce a multitude of secondary metabolites that play an eminent role in drug discovery, such as the blockbuster antibiotic penicillin G produced by *Penicillium notatum*.[1,2] In nature, microorganisms co-exist in complex communities, in which they interact with each other.[3,4] These interactions are mainly based on the production of secondary metabolites that are used as chemical signals for communication and/or competition for limited resources, favoring various defense mechanisms.[5] Therefore, mimicking the natural environment through mixed fermentation of different microorganisms (also called co-cultivation or co-culture) may lead to an enhancement in the production of compounds. In addition, co-cultivation may trigger the expression of silent biosynthetic pathways,[6,7] thus resulting in the accumulation of new natural products.[8,9] During our previous studies, co-cultivation of the endophytic fungus *Fusarium tricinctum* with the bacterium *Bacillus subtilis*, led to an up to 78-fold enhancement in the accumulation of the constitutively present fungal metabolites, along with the induction of new secondary metabolites that were not present in both fungal or bacterial axenic controls.[5] A similar observation was made when co-culturing the soil-dwelling fungus *Aspergillus terreus* with either *B. subtilis* or with *B. cereus*.[10] Besides the co-cultivation approach, treatment of fungi with epigenetic modifiers is another powerful tool for the activation of silent genes.[11] Recently, the addition of the DNA methyltransferase inhibitor 5-azacytidine to the endophytic fungus *Pestalotiopsis crassiuscula* resulted in a change in the secondary metabolite pattern and led to the induction of three new cryptic metabolites.[12]

During our ongoing search for new fungal secondary metabolites, we isolated the endophytic fungus *Chaetomium* sp. from fresh healthy leaves of *Sapium ellipticum* collected in Cameroon. Species of the genus *Chaetomium* have been described as endophytes [13] and are well known to produce a wide range of natural products such as the anticancer compounds chaetocochins A - C, [14] the antibacterial compound furano-polyene 3-*epi*-aureonitol, [15] and the metallo-β-lactamases inhibitors SB236050 and SB238569.[16, 17] Given this broad spectrum of bioactive secondary metabolites, we investigated a mixed-fermentation of *Chaetomium* sp. with the Gram-positive bacteria *B. subtilis* and *Streptomyces lividans*, as well as with *Mycobacterium tuberculosis*. In a further attempt to manipulate the pattern of fungal metabolites, *Chaetomium* sp. was treated with two epigenetic modifiers including the DNA methyltransferase inhibitor 5-azacytidine and the histone deacetylase inhibitor,

suberoylanilide hydroxamic acid (SAHA). The isolation and structure elucidation of the new metabolites (**8**-**12**) (Fig. 1), as well as the biological activities of the isolated compounds are described.

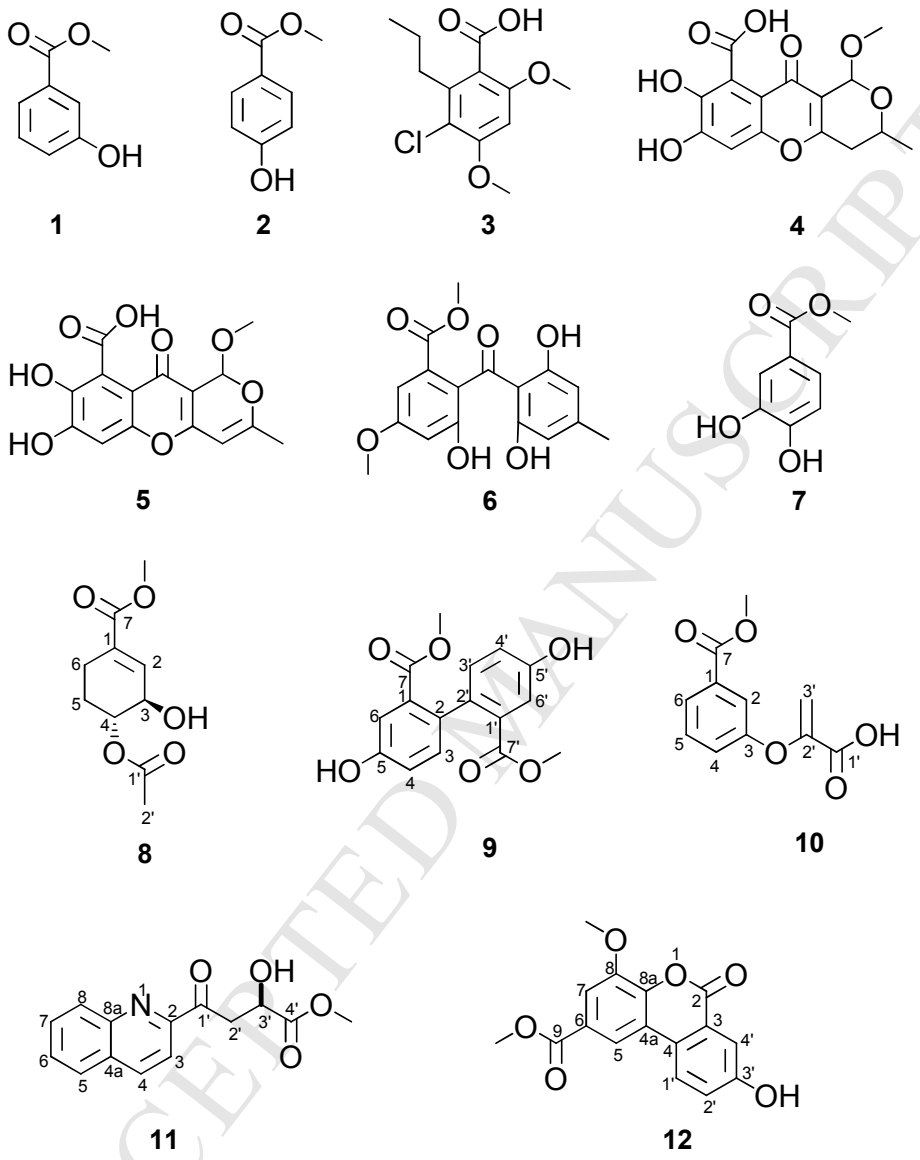

Figure 1. Structures of compounds **1**-**12**.

2. Results and discussion

Chaetomium sp. was isolated from the Cameroonian medicinal plant *Sapium ellipticum*. When *Chaetomium* sp. was cultured axenically on solid rice medium, average yields per culture flask were 2.8 mg for the known acremonisol A (**3**),[18] 13.9 mg for SB236050 (**4**),[16,17] 132.7 mg for SB238569 (**5**),[16,17] and 14.6 mg for the 1:1 mixture of 3- and 4-hydroxybenzoic acid methyl esters (**1** and **2**, respectively)[19,20] (Table 1). Co-cultivation of

Chaetomium sp. was undertaken with viable or autoclaved cultures of *B. subtilis*. In both cases, a strong accumulation of the 1:1 mixture of **1** and **2** was observed, accounting for a 8.3 and 7.4-fold increase, respectively, compared to axenic fungal controls (Table 1). However, the major polyketides SB236050 (**4**) and SB238569 (**5**), which are typical constituents of *Chaetomium* sp., were not detected in co-cultures (Fig. 2). These observations prove that the effects of co-cultivation are not uniform for all fungal metabolites, which is in agreement with our previously reported data.[5,10] In addition, seven compounds, including five new natural products (**8**-**12**), as well as the known isosulochrin (**6**) [21] and protocatechuic acid methyl ester (**7**) [22] were only detected in co-cultures of *Chaetomium* sp. with viable or with autoclaved *B. subtilis* cultures, but were lacking in axenic fungal or bacterial controls (Fig. 2).

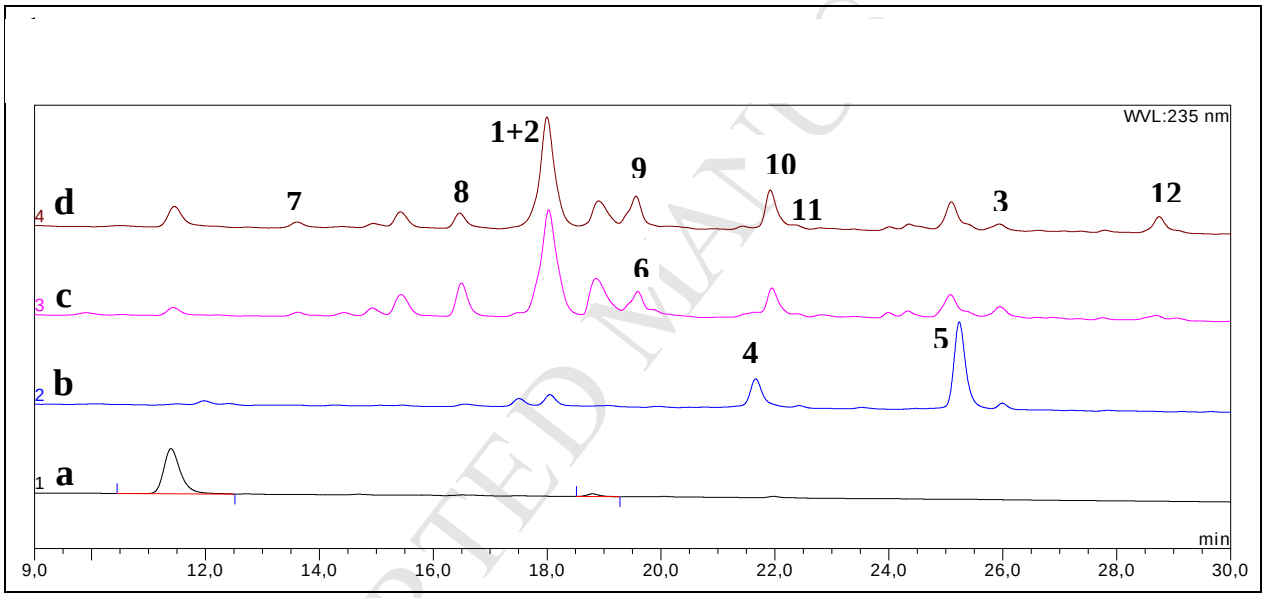

Figure 2. HPLC chromatograms of EtOAc extracts from co-culture experiments (detection at UV 235 nm): (a) *B. subtilis* control, (b) *Chaetomium* sp. control, (c) co-culture of *Chaetomium* sp. with autoclaved *B. subtilis*, (d) co-culture of *Chaetomium* sp. with viable *B. subtilis*.

Table 1: Yield of induced metabolites per flask during co-culture of *Chaetomium* sp. and *Bacillus subtilis* (n=5) vs. axenic controls of *Chaetomium* sp. (n=5).

Compound	Control[a] (mg)	*Chaetomium sp.* vs. *B. subtilis* (mg)	Increase (fold)	*Chaetomium sp.* vs. *autoclaved B. subtilis* (mg)	Increase (fold)
1+**2**	14.58 ± 5.88	121.20 ± 3.60	8.3	108.60 ± 7.20	7.4
3	2.82 ± 1.26	2.58 ± 0.90	0.9	1.86 ± 0.78	0.7

4	13.92 ± 0.07	n.d.	n.d.
5	132.72 ± 0.47	n.d.	n.d.
6	n.d.[b]	n.d.	26.82 ± 3.66
7	n.d.	3.06 ± 0.08	1.02 ± 1.44
8	n.d	4.98 ± 0.36	32.40 ± 5.52
9	n.d.	7.20 ± 0.96	n.d.
10	n.d.	13.98 ± 0.78	9.06 ± 0.18
11	n.d.	0.72 ± 0.12	n.d.
12	n.d.	19.20 ± 1.86	6.72 ± 0.36

[a] *Chaetomium* sp. axenic control. [b] n.d.: not detected

Compound **8** was isolated as a greenish oil. It exhibited a prominent ion peak at *m/z* 215.0913 $[M+H]^+$ in the HRESIMS spectrum, corresponding to the molecular formula $C_{10}H_{14}O_5$. Inspection of the ^{1}H NMR and COSY spectra of **8** revealed the presence of a methoxy group at δ_H 3.71 (3H, s, 7-OCH_3), a methyl group at δ_H 2.00 (3H, s, H_3-2'), as well as a continuous spin system composed of two methylene groups at δ_H 2.37 (2H, m, H_2-6) and 1.95/1.72 (1H, dddd, *J* = 13.2, 9.8, 8.2, 3.4 Hz, H_{2a}-5; 1H, dddd, *J* = 13.2, 9.8, 8.2, 6.5 Hz, H_{2b}-5), two oxymethine protons at δ_H 4.80 (1H, ddd, *J* = 9.0, 6.5, 3.4 Hz, H-4) and 4.27 (1H, dt, *J* = 9.0, 3.0 Hz, H-3), and an olefinic proton at δ_H 6.72 (1H, dt, *J* = 3.0, 1.9, H-2) (Table 2). In the HMBC spectrum of **8**, the correlations observed from 7-OCH_3, H-2, and H_2-6 to C-7 (δ_C 167.4) corroborated the attachment of the methoxy group (7-OCH_3) at C-7. Moreover, the HMBC correlations from H-4 and H_3-2' to C-1' (δ_C 170.8), suggested the presence of an acetyl group being located at C-4. These data were similar to those reported for methyl 4-*O*-acetyl-4-*epi*-shikimate,[23] the only difference being the absence of the hydroxy group at C-5 in **8**, which is in accordance with the 16 amu molecular weight difference between both compounds. The large coupling constant between H-3 and H-4 ($J_{3\text{-}4}$ = 9.0 Hz) indicated their *trans*-diaxial orientation, as reported for methyl 4-*O*-acetyl-4-*epi*-shikimate.[23]

Table 2: NMR data of **8** and **11**.

Position	Shikimeran A (**8**)[a]		Quinomeran (**11**)[b]	
	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)	δ_C, type[c]
1		131.8, C		
2	6.72, dt (3.0, 1.9)	139.4, C		153.6, C
3	4.27, dt (9.0, 3.0)	68.8, CH	8.10, d (8.4)	118.4, CH
4	4.80, ddd (9.0, 6.5, 3.4)	74.8, CH	8.53, d (8.4)	138.1, CH
4a				130.3, C
5	1.95, dddd (13.2, 9.8, 8.2, 3.4) 1.72, dddd (13.2, 9.8, 8.2, 6.5)	25.5,CH_2	8.08, dd (8.5, 1.5)	128.6,CH
6	2.37, m	23.5, CH_2	7.76, ddd (8.5, 6.9, 1.5)	129.5, CH
7		167.4, C	7.89, ddd (8.5, 6.9, 1.5)	131.0, CH
8			8.21, dd (8.5, 1.5)	130.9, CH
8a				147.7, C
1’		170.8, C		199.0, C
2’	2.00, s	21.2, CH_3	3.80, m	42.5, CH_2
3’			4.83, dd (6.9, 5.4)	67.9, CH
4’				174.5
4’-OCH_3			3.71, s	52.0
7-OCH_3	3.71, s	52.1, CH_3		

[a] Measured in $(CD_3)_2CO$ at 300 (^1H) and 75 (^{13}C) MHz.
[b] Measured in $(CD_3)_2CO$ at 600 (^1H) and 150 (^{13}C) MHz.
[c] Data extracted from HSQC and HMBC spectra.

For the determination of the absolute configuration of **8**, the solution TDDFT-ECD protocol [24] was carried out on the arbitrarily chosen (*3R,4R*)-**8** enantiomer. Merk Molecular Force (MMFF) conformational search in $CHCl_3$ resulted in 19 conformers in a 21 kJ/mol energy window. These conformers were reoptimized at B3LYP/6-31G(d) *in vacuo* and B97D/TZVP [25, 26] PCM/MeCN levels yielding 4 and 13 low-energy conformers above 2%, respectively (Figures S4 and 3, respectively). ECD spectra calculated at various levels (B3LYP/TZVP, BH&HLYP/TZVP and PBE0/TZVP *in vacuo*) for the individual conformers optimized in the gas-phase reproduced the ECD pattern of the experimental spectra, while the Boltzmann-average spectra did not resemble the experimental ECD. Although all conformers had similar computed ECD pattern at most levels, there was considerable wavelength deviation among the conformers, which caused the mismatch of the Boltzmann-weighted

ECDs. Conformers differed in the orientation of the methoxycarbonyl and the acetoxy groups (Figures S4 and S5).

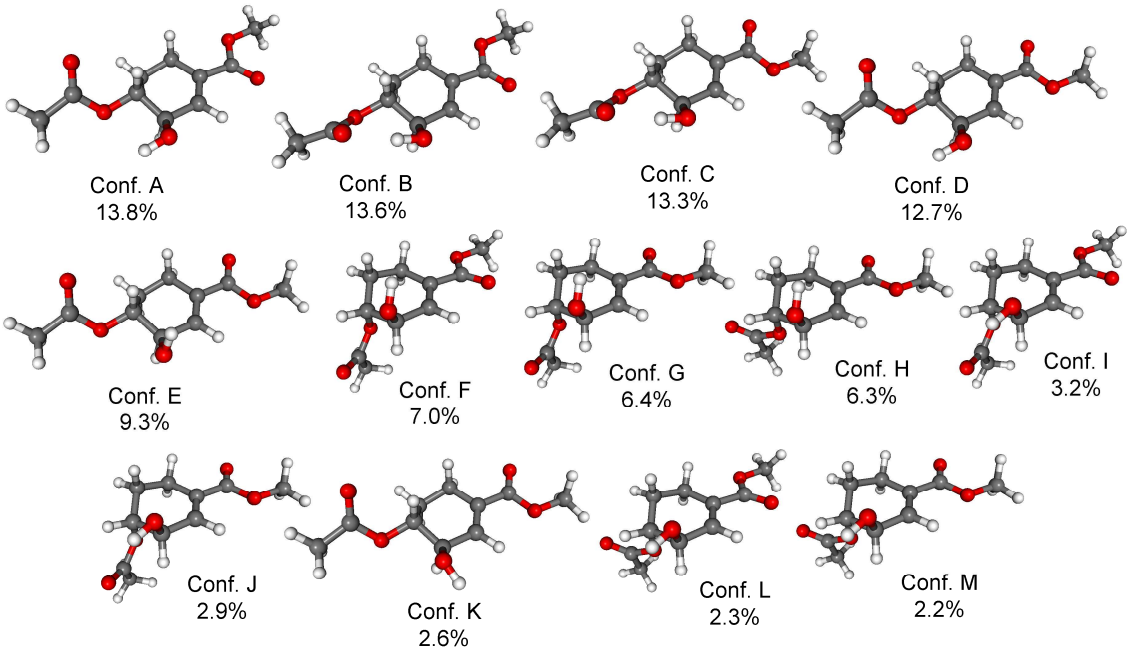

Figure 3. Structures and populations of the low-energy B97D/TZVP PCM/MeCN conformers (≥2%) of (3*R*,4*R*)-**8**.

In contrast, Boltzmann-weighted ECD spectra of (*3R,4R*)-**8** computed with PCM for MeCN at the same three levels for the B97D/TZVP PCM/MeCN reoptimized conformers gave nice agreement with the experimental ECD spectrum (Fig. 4) allowing the unambiguous determination of the absolute configuration as (*3R,4R*).

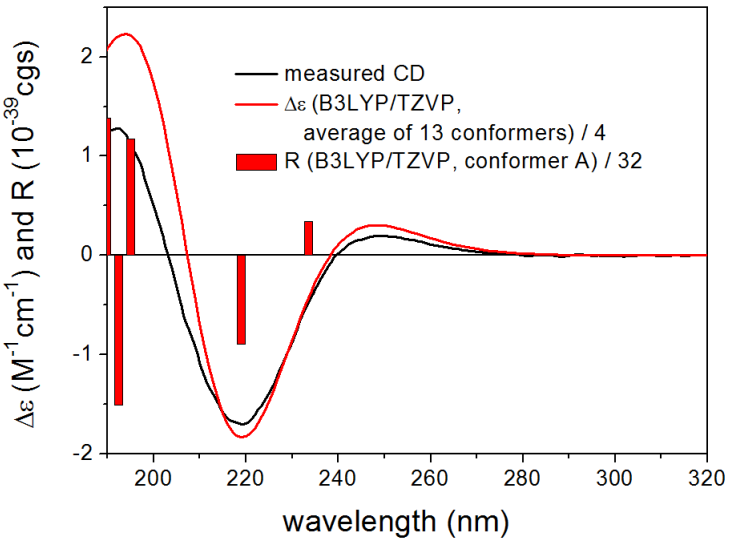

Figure 4. Experimental ECD spectrum of **8** in MeCN compared with the Boltzmann-weighted B3LYP/TZVP PCM/MeCN ECD spectrum of (3*R*,4*R*)-**8** computed for the B97D/TZVP PCM/MeCN conformers. Bars represent the rotational strength of the lowest-energy conformer.

Moreover, to exclude the possibility of **8** being an artefact formed during extraction with EtOAc, shikimic acid was incubated for 72h in EtOAc or MeOH at room temperature; however, no formation of the acetylated or methylated derivative, respectively, was observed by HPLC and LC-MS analysis. Thus, **8** was identified as a new natural product for which we propose the trivial name shikimeran A.

Compound **9** was isolated as a colorless oil. Its molecular formula was established as $C_{16}H_{14}O_6$ based on the prominent ion peak observed at *m/z* 303.0863 $[M+H]^+$ in the HRESIMS spectrum. Inspection of the ^1H and ^{13}C NMR spectra revealed only one set of five proton and eight carbon resonances, respectively (Table 3), indicating that **9** is a symmetrical dimer consisting of two identical monomers. Accordingly, the ^1H NMR spectrum (Table 3) of **9** displayed signals corresponding to six aromatic protons at δ_H 7.35 (2H, dd, J = 2.0, 1.1 Hz, H-6/6') and 7.02 (4H, H-3/3' and H-4/4'), two methoxy groups at δ_H 3.54 (6H, s, 7/7'-OMe), and two hydroxy protons at δ_H 8.65 (2H, br s, 5/5'-OH). In the HMBC spectrum of **2**, the correlations from H-4 (/4') to C-2 (/2') (δ_C 134.9) and C-6 (/6') (δ_C 116.9), from H-3 (/3') to C-5 (/5') (δ_C 157.0) and C-1 (/1') (δ_C 132.4), as well as from H-6 (/6') to C-4 (/4') (δ_C 119.1) and C-2 (/2') revealed the presence of a 1,2,4-trisubstituted phenyl ring (ABX spin system). In addition, the correlations from 5 (/5')-OH to C-4 (/4'), C-5 (/5'), and C-6 (/6') suggested the hydroxyl group being located at C-5 (/5'), adjacent to the aromatic protons H-4 (/4') and H-6

(/6'). This assignment was further corroborated by the ROESY correlations between 5 (/5')-OH and both H-4 (/4') and H-6 (/6'). Similarly, the HMBC correlations from 7/ (7')-OMe and H-6 (/6') to C-7 (/7') (δ_C 168.1) suggested that C-1 (/1') was substituted by a carbomethoxy group. The linkage between the two monomers was found to reside between C-2 and C-2', as H-3 (/3') displayed a strong HMBC correlation to C-2' (/2), allowing us to assign the planar structure of **9**. Notably, **9** was previously described as an intermediate in the total synthesis of aromatic polyesters containing multiple *n*-alkyl side chains.[27] However, to the best of our knowledge, this is the first report of the isolation of **9** from nature. Thus, **9** was identified as a new natural product for which the name bipherin A is proposed.

Table 3: NMR data of **9** measured in $(CD_3)_2CO$ at 300 (^{1}H) and 75 (^{13}C) MHz.

Position	Bipherin A (**9**)	
	δ_H, mult. (*J* in Hz)	δ_C, type
1/1'		132.4, C
2/2'		134.9, C
3/3'	7.02[a]	133.0, CH
4/4'	7.02[a]	119.1, CH
5/5'		157.0, C
6/6'	7.35, dd (2.0, 1.1)	116.9, CH
7/7'		168.1, C
7/7'-OCH_3	3.54, s	51.9, CH_3
5/5'-OH	8.65, br s	

[a] Signal overlap prevents determination of couplings.

Compound **10** was isolated as a yellowish oil. The HRESIMS spectrum exhibited a prominent ion peak at m/z 223.0602 $[M+H]^+$, indicating the molecular formula $C_{11}H_{10}O_5$. The ^{1}H NMR spectrum of **10** revealed the presence of four coupled signals at δ_H 7.77 (1H, dt, *J* = 8.0, 1.4 Hz, H-6), 7.61 (1H, dd, *J* = 2.5, 1.4 Hz, H-2), 7.47 (1H, t, *J* = 8.0 Hz, H-5), and 7.27 (1H, ddd, *J* = 8.0, 2.5, 1.4 Hz, H-4) (Table 4), indicating a 1,3-disubstituted benzene system. The remaining signals included those of a methoxy group at δ_H 3.90 (3H, s, 7-OCH_3) and two geminal olefinic protons at δ_H 5.83 (1H, d, *J* = 1.9 Hz, H_a-3') and 5.09 (1H, d, *J* = 1.9 Hz, H_b-3'), representing typical signals of a disubstituted vinyl group. The HMBC correlations from H-2, H-6, and 7-OCH_3 to C-7 (δ_C 167.8) suggested the presence of a methyl ester group at C-1. Moreover, the HMBC correlations from H_2-3' to C-1' (δ_C 165.7) and C-2' (δ_C 152.1), as well as the deshielded signal of the C-3 (157.8) were indicative of a monosubstituted acrylic acid moiety, which was connected to C-3 of the benzene ring through an ether bond. Thus, **10** was identified as a new natural product for which the name chorismeron is proposed.

Table 4: NMR data of **10** measured in CD_3OD at 500 (^1H) and 125 (^{13}C) MHz.

Position	Chorismeron (**10**)	
	δ_H, mult. (*J* in Hz)	δ_C, type
1		133.1, C
2	7.61, dd (2.5, 1.4)	119.9, CH
3		157.8, C
4	7.27, ddd (8.0, 2.5, 1.4)	124.4, CH
5	7.47, t (8.0)	131.1, CH
6	7.77, dt (8.0, 1.4)	125.7, CH
7		167.8, C
1’		165.7, C
2’		152.1, C
3’	5.09, d (1.9) 5.83, d (1.9)	107.2, CH_2
7-OCH_3	3.90, s	53.0, CH_3

Compound **11** was isolated as a brown oil. The HRESIMS spectrum exhibited a prominent ion peak at m/z 260.0915 $[M+H]^+$ consistent with the molecular formula $C_{14}H_{13}NO_4$. The ^1H NMR spectrum of **11** displayed two separate aromatic spin systems; the first consisted of the signals at δ_H 8.21 (1H, dd, *J* = 8.5, 1.5 Hz, H-8), 8.08 (1H, dd, *J* = 8.5, 1.5 Hz, H-5), 7.89 (1H, ddd, *J* = 8.5, 6.9, 1.5 Hz, H-7), and 7.76 (1H, ddd, *J* = 8.5, 6.9, 1.5 Hz, H-6), typical of a disubstituted aromatic ring (ABCD spin system) (Table 2). The second spin system consisted of two *ortho* protons at δ_H 8.53 (1H, d, *J* = 8.4 Hz, H-4) and 8.10 (1H, d, *J* = 8.4 Hz, H-3) (Table 2). These signals were indicative of a quinoline moiety, as confirmed by the HMBC correlations from H-5 to C-7 (δ_C 131.0), C-8a (δ_C 147.7), and C-4 (δ_C 138.1), from H-8 to C-6 (δ_C 129.5) and C-4a (δ_C 130.3), and from H-4 to C-5 (δ_C 128.6), C-8a, and C-2 (δ_C 153.6) (Fig. 5). The remaining signals of a methylene group resonating at δ_H 3.80 (2H, m, H_2-2’), an oxymethine proton at δ_H 4.83 (1H, dd, *J* = 6.9, 5.4 Hz, H-3’) and a methoxy signal at δ_H 3.71 (3H, s, 4’-OCH_3) were attributed to a 2-hydroxy-4-oxobutanoic acid methyl ester moiety, as supported by the COSY correlations between H_2-2’ and H-3’, as well as by the HMBC correlations from 4’-OCH_3 to C-3’(δ_C 67.9) and C-4’ (δ_C 174.5), and from both H_2-2’ and H-3’to C-1’ (δ_C 199.0) and C-4’ (Fig. 5). Finally, the HMBC correlation from H-3 to C-1’ corroborated the attachment of the 2-hydroxy-4-oxobutanoic acid methyl ester moiety at C-2 of the quinoline ring. The baseline ECD spectrum of **11** in acetonitrile indicated that it is a racemic mixture (Figure S20). Thus, **11** was identified as a new natural product for which the name quinomeran is proposed.

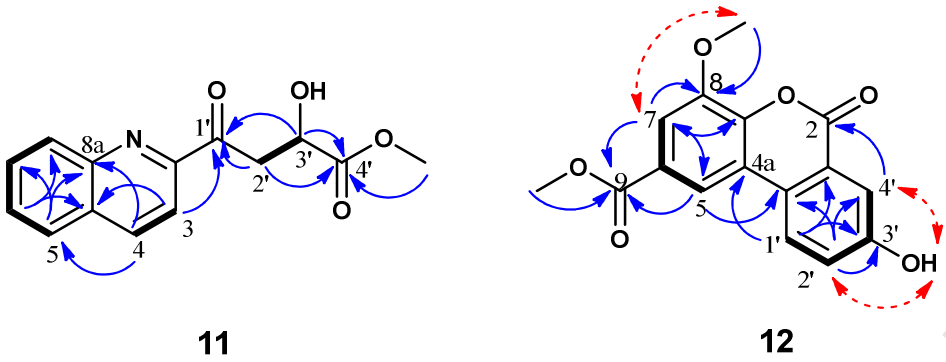

Figure 5. COSY (bold), key ROESY (dashed), and HMBC (plain) correlations of **11** and **12**

Compound **12** was isolated as a yellow amorphous powder. The molecular formula of **12** was determined as $C_{16}H_{12}O_6$ based on the prominent ion peak at m/z 301.0706 $[M+H]^+$. It exhibited UV absorption maxima at 202, 236, and 361 nm, typical for dibenzo-α-pyrone derivatives. Inspection of the ^{1}H NMR (Table 5) of **12** indicated the presence of two meta-coupled protons at δ_H 8.27 (1H, d, J = 1.9 Hz, H-5) and 7.55 (1H, d, J = 1.9 Hz, H-7), as well as an ABX-type spin system, consisting of three aromatic protons at δ_H 8.28 (1H, d, J = 8.8 Hz, H-1'), δ_H 7.37 (1H, dd, J = 8.8, 2.7 Hz, H-2'), and δ_H 7.56 (1H, d, J = 2.7 Hz, H-4'). Additional signals included those of two methoxy groups at δ_H 3.96 (3H, s, 8-OCH_3) and 3.90 (3H, s, 9-OCH_3), and a phenolic hydroxy proton at δ_H 10.55 (1H, s, 3'-OH). Further detailed analysis of the 2D NMR (COSY, HSQC, and HMBC) spectra allowed us to establish the substitution pattern of the dibenzo-α-pyrone skeleton of **12**, as shown in Figure 1. Accordingly, the position of the hydroxy group (3'-OH) was assigned at C-3', as it showed ROESY correlations to H-2' and H-4' (Fig. 5). Moreover, the HMBC correlations from 9-OCH_3, H-7, and H-5 to C-9 (δ_C 165.6) corroborated the presence of a carbomethoxy group and its attachment at C-6. Finally, the remaining methoxy group (8-OCH_3) was assigned at C-8 (δ_C 147.3), as supported by the respective HMBC correlation, as well as by its ROESY correlation with H-7 (Figure 2). Thus, **12** was identified as a new natural product for which the name serkydayn is proposed.

Table 5: NMR data of **12** measured in DMSO-*d6* at 300 (^{1}H) and 75 (^{13}C) MHz.

Position	Serkydayn (**12**)	
	δ_H, mult. (*J* in Hz)	δ_C, type
1		
2		159.4, C
3		118.7, C
4		125.1, C
4a		121.9, C
5	8.27, d (1.9)	115.5, CH
6		125.7, C
7	7.55, d (1.9)	110.8, CH
8		147.3, C
8a		142.1, C
1’	8.28, d (8.8)	125.1, CH
2’	7.37, dd (8.8, 2.7)	124.1, CH
3’		158.8, C
4’	7.56, d (2.7)	113.8, CH
9		165.6, C
9-OMe	3.90, s	52.4, CH_3
8-OMe	3.96, s	56.1, CH_3
3’-OH	10.55, s	

The new metabolites **8**, **9**, **10**, and **12** probably originate from *Chaetomium* sp. based on structural analogies with the known metabolites (**1**-**7**), which were obtained from the axenic fungal control. Compound, **11** is the only quinoline derivative in this series of metabolites, which highlights the value of the co-cultivation approach as a powerful tool to activate silent biosynthetic gene clusters in microorganisms. Quinolines produced by fungi have already been reported,[28,29] and thus the fungal origin of **11** is likely. Interestingly, compounds **8**, **10** and **12** were detected during co-cultivation of *Chaetomium* sp. with viable or autoclaved cultures of *B. subtilis*, whereas compounds **9** and **11** were only detected during co-cultivation of *Chaetomium* sp. with viable *B. subtilis* cultures. These data indicate that the effect of heat sterilized bacterial biomass is not uniform for all fungal metabolites.

It is worth mentioning that the production of fungal metabolites in the co-cultures was found to correlate with the time of preincubation of the solid rice medium with *B. subtilis* prior to inoculation with *Chaetomium* sp.. The strongest effect was observed when the fungus was added 4 days after the rice medium had been inoculated with viable *B. subtilis* (data not shown). During co-cultivation, the fungal growth was slowed down compared to axenic controls of *Chaetomium* sp., but recovered after seven to nine days of co-culture. These data demonstrate an inhibitory effect due to the presence of the bacterium, which is in agreement with our previously reported results.[5]

In a second set of experiments, the endophytic bacterium *Streptomyces lividans*[30] was chosen for co-cultivation with *Chaetomium* sp.. However, no induction of fungal metabolites was detected. Likewise, no induction of fungal metabolites was observed when *Chaetomium* sp. was co-cultured with autoclaved *Mycobacterium smegmatis* (data not shown). These results suggest that the fungal response is specific toward different prokaryotes, as previously also described for other fungi.[5,10]

Taking into consideration that posttranslational modifications of histones influence the pattern of secondary metabolites in filamentous fungi,[31] *Chaetomium* sp. was cultured on solid rice medium in the presence of the DNA methyltransferase inhibitor 5-azacytidine or the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). The HPLC chromatographic profiles of the respective extracts were significantly different in comparison to those of the axenic fungal controls, but were very similar to each other and showed the induction of two new peaks, which were not present in the fungal controls (Figure S25). Interestingly, one of the induced peaks was identified as the known isosulochrin (**6**), which was also detected during co-cultivation of *Chaetomium* sp. with *B. subtilis*. Thus, the effect on the accumulation of fungal metabolites during co-cultivation may be partially triggered by histone modifications due to microbial crosstalk.

All compounds isolated in this study were assayed *in vitro* for their antibacterial activities against the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, and *Bacillus subtilis* 168 trpC2; the latter being the bacterial strain used for co-cultivation. The compounds were likewise assayed against the Gram-negative bacterium *Acinetobacter baumannii*, as well as against *Mycobacterium tuberculosis*. Compound **12** exhibited weak to moderate activity against *B. subtilis* with an MIC value of 53 μM, whereas the remaining compounds exhibited no activity (MIC > 100 μM). In addition, all compounds were evaluated for their effects on the growth of the mouse lymphoma L5178Y cell line. Compound **12** likewise showed the strongest activity with an IC_{50} value of 1 μM. Compounds **11** and **7** displayed only weak cytoxicity with IC_{50} values of 38.6 and 20.8 μM, respectively. Interestingly, the biosynthesis of **12** was induced only during fungal/bacterial co-cultivation, and thus it could be assumed that *Chaetomium* sp. initiated its production as a stress response to suppress its competitor.

3. Conclusion

In conclusion, the axenic culture of *Chaetomium* sp. grown on solid rice medium yielded five known metabolites (**1 ─ 5**). When *Chaetomium* sp. was grown in mixed cultures with viable or autoclaved cultures of *B. subtilis*, a strong accumulation of the 1:1 mixture of **1** and **2** was observed compared to axenic fungal or bacterial controls. In addition, five new natural products (**8 ─ 12**) together with **6** and **7** were only detected in the co-cultures. Likewise, induction of **6** was observed when *Chaetomium* sp. was cultured on solid rice medium with the epigenetic modifiers 5-azacytidine or SAHA. Thus, our results highlight the potential of the co-cultivation and epigenetic modification as powerful strategies for triggering the production of cryptic fungal secondary metabolites.

4. Experimental Section

4.1. General Experimental Procedures

Optical rotations were determined on a Perkin-Elmer-241 MC polarimeter. ^{1}H, ^{13}C and 2D NMR spectra were recorded in deuterated solvents on a Bruker ARX 300, Avance III 500, Avance DMX 600 or AV III HD 700 NMR spectrometers. Mass spectra were obtained on a LC-MS HP1100 Agilent Finnigan LCQ Deca XP Thermoquest and high-resolution mass (HRESIMS) spectra were measured on a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. HPLC analysis was performed with a Dionex UltiMate3400 SD with a LPG-3400SD Pump coupled to a photodiode array detector (DAD3000RS); routine detection was at 235, 254, 280, and 340 nm. The separation column (125 mm × 4 mm) was prefilled with Eurosphere-10 C18 (Knauer, Germany), and the following gradient was used (MeOH, 0.1 % HCOOH in H_2O): 0 min (10 % MeOH), 5 min (10% MeOH), 35 min (100 % MeOH), 45 min (100 % MeOH). Semi-preparative HPLC was performed using a Merck Hitachi HPLC System (UV detector L-7400; Pump L-7100; Eurosphere-100 C18, 300 mm × 8 mm, Knauer, Germany). Column chromatography included LH-20 Sephadex and Merck MN Silica gel 60 M (0.04-0.063 mm). TLC plates with silica gel F254 (Merck, Darmstadt, Germany) were used to monitor fractions (CH_2Cl_2/MeOH mixtures as mobile phase); detection was under UV at 254 and 366 nm or by spraying the plates with anisaldehyde reagent. Bacterial growth was monitored by measuring OD_{600} in a Tecan microtiter plate reader (Infinite M200, Tecan).

4.2. Biological material

Chaetomium sp. was isolated from fresh healthy leaves of *Sapium ellipticum* (Euphorbiaceae) collected in the west region of Cameroon in January 2015. The fungus was isolated under sterile conditions from the inner tissue of the leaf according to the procedure described by Kjer et al.[32] The identification was performed following a molecular biological protocol by DNA amplification and sequencing of the ITS region. The sequence data have been submitted to GenBank, accession number **KU051539**. The bacterial strain panel used on this study included the standard laboratory strains, *B. subtilis* 168 trpC2,[33] *S. lividans* TK24,[34] and *M. smegamatis* (mc^2 155).[35]

4.3. Co-cultivation experiment of *Chaetomium* sp. with *B. subtilis* 168 trpC2

The fungal and bacterial strains were cultivated in Erlenmeyer flask (1 L) containing solid rice media for isolation and characterization of secondary metabolites. Twenty-five Erlenmeyer flasks (five flasks for axenic *Chaetomium* sp., five for co-cultures of *Chaetomium* sp. and *B. subtilis*, five for *Chaetomium* sp. treated with autoclaved *B. subtilis*, five for axenic *B. subtilis* and five for autoclaved *B. subtilis*) containing 60.0 mL of distilled water and 50.0 g of commercially available milk rice (Milch-Reis, ORYZA) each were autoclaved before the fungus and the bacterium were inoculated.

The bacterium *B. subtilis* was grown in lysogeny broth (LB). An overnight culture of this bacterium was used to inoculate prewarmed LB medium (1:20), which was afterwards incubated at 37°C with shaking at 200 rpm to mid exponential growth phase (optical density at 600 nm (OD_{600}) of 0.2-0.4). A 10 mL volume of the bacterial culture was then added to the rice medium, which was further incubated for 4 days at 37°C.

This preincubation was followed by the addition of *Chaetomium* sp. grown on malt agar (three pieces, 1 cm-1 cm) to the rice medium containing viable *B. subtilis*. On the other hand, after 4 days incubation, ten flasks cultured with *B. subtilis* were autoclaved followed by addition of the fungus to five of them, in the same manner like for viable cultures of *B. subtilis*. Both microorganisms were also grown axenically on rice medium. Co-cultures and axenic cultures of *Chaetomium* sp. and *B. subtilis* were kept under static conditions at 23°C until they reached their stationary phase of growth (3 weeks for *Chaetomium* sp. and *B. subtilis*; 5 weeks for co-cultures). For extraction, 300 mL of EtOAc was added to the cultures, and then the resulting mixture was shaken at 140 rpm for 9 h. The cultures were further left

overnight and filtered on the following day using a Buchner funnel. The resulting extract was washed with demineralised water and then evaporated under vacuum to remove EtOAc. Each extract was then dissolved in 60 mL of MeOH, and then 15 μL of this was injected into the analytical HPLC column.

4.4. Co-cultivation experiment of *Chaetomium* sp. with *S. lividans* TK24

The co-cultivation of *Chaetomium* sp. with *S. lividans* was done in the same manner as with viable *B. subtilis*. Fifteen Erlenmeyer flasks (five for axenic *Chaetomium* sp., five for *Chaetomium* sp. and *S. lividans* and five for axenic *S. lividans*) containing 60.0 mL of Yeast Malt (YM) medium and 50.0 g of commercially available milk rice (Milch-Reis, ORYZA) each were autoclaved before inoculating the fungus and the bacterium. An overnight culture of *Streptomyces lividans* was used to inoculate prewarmed YM medium (1:20), which was then incubated at 30°C with shaking at 200 rpm to mid exponential growth phase. The corresponding preculture was afterwards incubated in fresh YM medium overnight to reach exponential growth phase. Then 10 mL volume of the bacterial culture was added to the rice medium, which was further incubated for four days at 30°C. After this preincubation, we proceeded in the same manner as described in the co-cultivation experiment of *Chaetomium* sp. and *B. subtilis* 168 trpC2.

4.5. Co-cultivation experiment of *Chaetomium* sp. with *Mycobacterium smegmatis* (mc^2 155)

The fungal and the bacterial strains were cultivated in Erlenmeyer flask (1L) containing solid rice media for isolation and characterization of secondary metabolites. Erlenmeyer flasks containing 60.0 mL of distilled water and 50.0 g of commercially available milk rice (Milch-Reis, ORYZA) each were autoclaved before the fungus and the bacterium were inoculated. *M. smegmatis* was grown in Middelbrook 7H9 media supplemented with 0.5% (v/v) glycerol, 0.05% (v/v) tyloxapol and 10% (v/v) ADS enrichment (5%, w/v, bovine serum albumin fraction V; 2%, w/v, glucose; 0.85%, w/v, sodium chloride) until exponential growth phase (OD 600 nm of 0.5-0.8). 10 mL volume of the bacterial culture was then added to the rice medium, which was further incubated for 4 days 37°C and finally autoclaved. After this preincubation, we proceeded in the same manner as described in the co-cultivation experiment of *Chaetomium* sp. and *B. subtilis* 168 trpC2.

4.6. Treatment with epigenetic modifiers

The treatment of *Chaetomium* sp. with the histone deacetylase inhibitor SAHA or with DNA methyltranferase inhibitor 5-azacytidine, was carried out to compare the production of cryptic fungal metabolites induced during the co-culture experiments with fungal cultures being treated with epigenetic modifications. Ten flasks (two flasks for axenic *Chaetomium* sp., two flasks for *Chaetomium* sp. and SAHA, two flasks for *Chaetomium* sp. and 5-azacytidine, two flask of SAHA and two flask of 5-azacytidine) containing 60.0 mL of distilled water and 50.0 g of commercially available milk rice (Milch-Reis, ORYZA) each were autoclaved before inoculation of the fungus and addition of the epigenetic modifiers. Three pieces of *Chaetomium* sp. grown on malt agar were added to the autoclaved rice medium under sterile conditions and incubated for two days. This preincubation was followed by the addition of 10 mL of the solution (Sterilized Water-EtOH 21:4) of each epigenetic modifier (6 mM each) under sterile conditions. Epigenetic and axenic cultures of *Chaetomium* sp. were kept under static conditions at 23°C until they reached their stationary phase of growth (3 weeks for *Chaetomium* sp.; 4 weeks for epigenetic cultures). After this incubation, we proceeded in the same manner as described in the co-cultivation experiments of *Chaetomium* sp. and *B. subtilis* 168 trpC2.

4.7. Extraction and isolation

The crude extract obtained from co-cultures of *Chaetomium* sp. with *B. subtilis* (6.0 g) was subjected to vacuum liquid chromatography (VLC) on silica gel employing a step gradient of *n*-hexane-EtOAc then CH_2Cl_2-MeOH to give seven fractions A-G. Fraction B, eluted with *n*-hexane/EtOAc (6:4) was subjected to repeated VLC, using only a step gradient of *n*-hexane-EtOAc to yield nine subfractions B_1-B_9. Subfraction B_2, eluted with *n*-hexane-EtOAc (9:1, v/v), was further purified using Sephadex LH-20 followed by semipreparative HPLC with MeOH-H_2O (0.1%TFA) to afford the mixture **1** and **2** (50 mg). Subfraction B_3, eluted with *n*-hexane-EtOAc (8.5:1.5, v/v), was purified by semipreparative HPLC with MeOH-H_2O (0.1%TFA) to yield **11** (1.1 mg) and **7** (1.2 mg). Subfraction B_4, eluted with *n*-hexane-EtOAc (8:2, v/v), was further purified using Sephadex LH-20 followed by semipreparative HPLC with MeOH-H_2O to yield **8** (11 mg), **12** (6 mg) and **6** (4 mg). Subfraction B_5, eluted with *n*-hexane-EtOAc (7.5:2.5, v/v), was treated in the same manner as B_4 to afford **9** (8 mg), **10** (2 mg) and **3** (3.8 mg).

The crude extract of the axenic culture of *Chaetomium* sp. (1.6 g) was also subjected to VLC on silica gel employing a step gradient of *n*-hexane-EtOAc followed by CH_2Cl_2-MeOH to give six fractions A-F. Fraction E, eluted with EtOAc, was purified by semipreparative HPLC (MeOH-H_2O) to yield **4** (1.9 mg) and **5** (3.1 mg). Compounds **1**, **2**, **3**, **4**, **5**, **6**, and **7** were identified by comparing their ^{1}H NMR, LC-MS and UV data with those published.[16,18-22]

The crude extracts resulting from co-cultivation of *Chaetomium* sp. with *S. lividans* or with *M. smegmatis* as well as those from the epigenetic modifier experiments were directly submitted to analytical HPLC and no further work up was undertaken due to their limited amounts.

4.7.1. Shikimeran A (**8**). Greenish oil; $[\alpha]^{20}_D$ -58 (*c* 1.01, MeOH); UV (MeOH) λ_{max}: 227 nm; ECD (MeCN,λ [nm] (Δε), *c* = 3.50x10^{-4}M): 250 (0.20), 220 (-1.72), 192 (1.29); ^{1}H and ^{13}C NMR see Table 2; HRESIMS $[M+H]^+$ m/z 215.0913 (calcd for $C_{10}H_{15}O_5$, 215.0914).

4.7.2. Bipherin A (**9**). Colorless oil; UV (MeOH) λ_{max}: 233 and 307 nm; ^{1}H and ^{13}C NMR see Table 3; HRESIMS $[M+H]^+$ m/z 303.0863 (calcd for $C_{16}H_{15}O_6$, 303.0863).

4.7.3. Chorismeron (**10**)**.** Yellowish oil; UV (MeOH) λ_{max}: 205 and 289 nm; ^{1}H and ^{13}C NMR see Table 4; HRESIMS $[M+H]^+$ m/z 223.0602 (calcd for $C_{11}H_{11}O_5$, 223.0601).

4.7.4. Quinomeran (**11**)**.** Brown oil; $[\alpha]^{20}_D$ 0 (*c* 0.3, MeOH); UV (MeOH) λ_{max}: 208, 246, and 297 nm; ^{1}H and ^{13}C NMR see Table 2; HRESIMS $[M+H]^+$ m/z 260.0915 (calcd for $C_{14}H_{14}NO_4$, 260.0917).

4.7.5. Serkydayn (**12**)**.** Yellow amorphous powder; UV (MeOH) λ_{max}: 202, 236, and 361 nm; ^{1}H and ^{13}C NMR see Table 5; HRESIMS $[M+H]^+$ m/z 301.0706 (calcd for $C_{16}H_{13}O_6$, 301.0707).

4.8. Computational section

Mixed torsional/low-frequency mode conformational searches were carried out by means of the Macromodel 9.9.223 software using the Merck Molecular Force Field (MMFF) with an implicit solvent model for $CHCl_3$.[36] Geometry reoptimizations were carried out at the

B3LYP/6-31G(d) level *in vacuo* and the B97D/TZVP level [25, 26] with the PCM solvent model for MeCN. TDDFT ECD calculations were run with various functional (B3LYP, BH&HLYP, PBE0) and the TZVP basis set as implemented in the Gaussian 09 package with the same or no solvent model as in the preceding DFT optimization step.[37] ECD spectra were generated as sums of Gaussians with 3000 cm^{-1} widths at half-height (corresponding to ca. 15 nm at 220 nm), using dipolevelocity-computed rotational strength values.[38] Boltzmann distributions were estimated from the ZPVE-corrected B3LYP/6-31G(d) energies in the gas-phase calculations and from the B97D/TZVP energies in the solvated ones. The MOLEKEL software package was used for visualization of the results.[39]

4.9. Antibacterial assay

Measurement of MIC values were done by the broth microdilution method according to CLSI guidelines.[40] The direct colony suspension method was used with an inoculum of 5 x 10^5 colony forming units/mL after the last dilution step for inoculum preparation. Compounds were added from stock solution (10 mg/mL in DMSO), resulting in a final DMSO amount of 0.64% at the highest antibiotics concentration tested (64 μg/mL). Serial 2-fold dilutions of antibiotics were prepared with DMSO being diluted along with the compounds.

4.10. Antituberculosis assay

The resazurin dye reduction method was performed as a metabolic assay to evaluate the growth inhibition of *M. tuberculosis*. *M. tuberculosis* cells were grown aerobically at 37°C in Middlebrook 7H9 media supplemented with 0.5% (v/v) glycerol, 0.05% (v/v) Tyloxapol, and 10% (v/v) ADS enrichment (5%,w/v, bovine serum albumin fraction V; 2%, w/v, glucose; 0.85%, w/v, sodium chloride). Bacteria were precultured until log-phase (OD 600 nm~1) and then seeded at 1 x 10^5 cells per well in a total volume of 100 μL in 96-well round-bottom microtiter plates and incubated with test substances for 6 days. For viability determination, 10 μL of resazurin solution (100 μg/mL, Sima-Alderich) was added per well and incubated for ca. 8h. Then cells were fixed at room temperature for 30 mn after addition of formalin (5%, v/v, final concentration), and fluorescence was measured using a microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to rifampicin-treated (0% growth) and DMSO treated (100% growth) controls.

4.11. Cell viability assay

Cytotoxicity was tested against L5178Y mouse lymphoma cells using an MTT (=3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay and compared to that of untreated controls, as described previously.[41] Experiments were repeated three times and carried out in triplicate. As negative controls, media with 0.1% EGMME/ DMSO were included in the experiments. The depsipeptide kahalalide F, isolated from *Elysia grandifolia* was used as a positive control.

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Figure Legends

Figure 1. Structures of compounds **1-12**.

Figure 2. HPLC chromatograms of EtOAc extracts from co-culture experiments (detection at UV 235 nm): (a) *B. subtilis* control, (b) *Chaetomium* sp. control, (c) co-culture of *Chaetomium* sp. with autoclaved *B. subtilis*, (d) co-culture of *Chaetomium* sp. with viable *B. subtilis*.

Figure 3. Structures and populations of the low-energy B97D/TZVP PCM/MeCN conformers (≥2%) of (3*R*,4*R*)-**8**.

Figure 4. Experimental ECD spectrum of **8** in MeCN compared with the Boltzmann-weighted B3LYP/TZVP PCM/MeCN ECD spectrum of (3*R*,4*R*)-**8** computed for the B97D/TZVP PCM/MeCN conformers. Bars represent the rotational strength of the lowest-energy conformer.

Figure 5. COSY (bold), key ROESY (dashed), and HMBC (plain) correlations of **11** and **12**.