Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 13 (2005) 5267–5273
Bioorganic & Medicinal Chemistry

Libertellenones A–D: Induction of cytotoxic diterpenoid biosynthesis by marine microbial competition

Dong-Chan Oh, Paul R. Jensen, Christopher A. Kauffman and William Fenical*

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0204, USA

Received 1 April 2005; revised 30 May 2005; accepted 30 May 2005
Available online 1 July 2005

Abstract—The induction of biosynthesis of four new diterpenoids was observed following the addition of a marine α-proteobacterium to an established culture of the marine-derived fungus *Libertella* sp. The fungal strain and the marine bacterium, cultured alone, do not produce diterpenoid metabolites. The induced diterpenoids, libertellenones A–D, are cross-conjugated ketones of the pimarane class. The libertellenones show varying levels of cytotoxicity against the HCT-116 human adenocarcinoma cell line with libertellenone D being the most potent (IC_{50} = 0.76 μM).

1. Introduction

Microbial competition for limited space and nutrients is thought to be the major ecological force driving the production of bioactive secondary metabolites.[1,2] This type of competitive interaction, mediated by antibiotic production, has long been recognized among soil bacteria[3] and now has also been documented among pelagic marine bacteria.[4] It has been hypothesized that the secondary metabolites responsible for the antagonistic effects may only be produced in response to interactions with other microbes. Thus, culturing different microbes together (co-culture) forces direct interactions that may induce the production of compounds not previously observed when strains are cultured independently. The application of this co-culture strategy represents a potentially important approach to the discovery of novel secondary metabolites.[4]

Most previous studies using co-culture techniques have provided evidence for the induction of antibiotic biosynthesis but have not led to the induction of specific metabolic pathways. For example, it has been shown that antibiotic activity increased in crude extracts when microbial strains were cultured together.[5–7] Similarly, increased yields of some previously described metabolites have been observed in co-culture competition experiments.[8] These include new analogues of lipoaminopeptides produced in a mixed culture of *Acremonium* sp. and *Mycogone rosea*.[9] Increased yields of the antibiotic istamycin have also been reported from the marine bacterium *Streptomyces tenjimariensis* when this strain was co-cultured with unicellular marine bacteria.[10] However, as far as we are aware, the sole example of the induction of a previously unexpressed biosynthetic pathway in response to co-cultivation is pestalone biosynthesis in the marine-derived fungal strain *Pestalotia* sp.[11]

Here, we report another example of the induction of a biosynthetic pathway, this time diterpene biosynthesis, in response to microbial co-culture interactions. In this case, a series of new pimarane diterpenoids, libertellenones A–D, were produced in response to the addition of a marine α-proteobacterium (strain CNJ-328) to an established 3-day-old culture of a marine-derived fungus identified as a *Libertella* sp. These new compounds were not observed in pure cultures of the fungus or the bacterium and are proposed to be of fungal origin. Curiously, the marine bacterium CNJ-328, which is capable of inducing diterpenoid biosynthesis, is the same strain that induced pestalone production in the previously mentioned study.[11]

2. Results and discussion

Because fungi and bacteria have been observed in both synergistic and antagonistic relationships,[12] these

Keywords: Co-culture; Induction of biosynthesis; Libertellenones.
* Corresponding author. Tel.: +1 858 534 2133; fax: +1 858 558 3702; e-mail: wfenical@ucsd.edu

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.05.068

interactions may induce the production of secondary metabolites that are not observed when the strains are grown in pure culture. To test this hypothesis, we used a co-culture method to screen for the induction of secondary metabolite biosynthesis. More than 50 fungal strains were challenged by adding a marine bacterium (CNJ-328) to an established fungal fermentation and monitored for secondary metabolite production by LC/MS in comparison to control cultures of the fungi and the bacterium CNJ-328 alone. In most cases, only slight modifications of metabolites in mixed cultures were noted. This was not the case, however, with the co-culture of fungal strain CNL-523, a *Libertella* sp., and the marine bacterium CNJ-328. When a small volume (1 mL) of bacterial culture was added to an established 1 L culture of fungal strain CNL-523, four new peaks in the LC/MS trace were rapidly (within 24 h) observed. These compounds were subsequently isolated and identified as a series of new pimarane diterpenes, libertellenones A–D (**1**–**4**, Fig. 1). The structures of the libertellenones, including their absolute stereostructures, were assigned by combined chemical and spectral methods (Fig. 2).

Libertellenone A (**1**) was isolated as white amorphous powder, which was analyzed for the molecular formula $C_{20}H_{28}O_4$ (seven unsaturation equivalents) by HR-

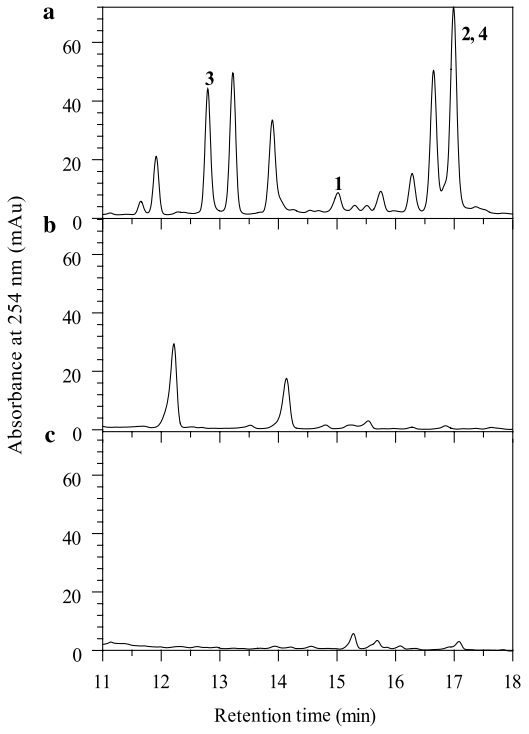

Figure 1. Gradient LC/MS traces (detected at 254 nm) of (a) the co-culture extract (*Libertella* sp. + CNJ-328), (b) the pure fungal culture extract (*Libertella* sp.), and (c) the pure bacterial culture extract (CNJ-328), all taken from 4-day-old cultures. For the co-culture (a), day 4 is 24 h after the inoculation of the bacterium. Libertellenone D (**4**) was found to co-elute with libertellenone B (**2**). The conditions of the LC/MS run were: analytical C-18 reversed-phase column, eluting with a CH_3CN/H_2O gradient solvent system from 10% aqueous CH_3CN to 100% CH_3CN over 30 min. The LC/MS samples were prepared at 1 mg/mL of crude extract and 20 μL of each sample was injected.

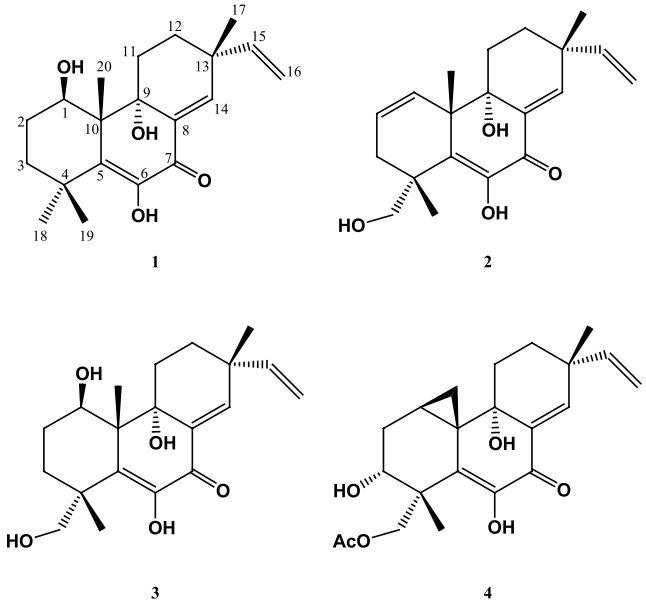

Figure 2. Structures of the libertellenone A (**1**), B (**2**), C (**3**), and D (**4**).

MALDI mass spectrometry (obsd $[M+Na]^+$ at *m/z* 355.1885, calcd $[M+Na]^+$ 355.1886) in combination with ^1H and ^{13}C NMR spectral data (Table 1). The ^1H NMR spectrum of **1** demonstrated signals assigned to a terminal vinyl group [δ_H: 5.84 (dd, 17.5, 10.5); 5.07 (dd, 17.5, 1.0); 5.03 (dd, 10.5, 1.0)], one secondary alcohol [δ_H : 4.34 (dd, 9.5, 5.5)], and four tertiary methyl groups [δ_H: 1.38 (s); 1.28 (s); 1.19 (s); 1.11 (s)]. Analysis of ^{13}C NMR data showed one a,b-unsaturated carbonyl carbon [δ_C: 181.3], six olefinic carbons [δ_C: 149.1; 145.4; 145.1; 143.5; 133.2; 112.5], two oxygenated sp^3 carbons [δ_C: 74.6; 69.3], and 11 aliphatic carbons between 20 and 50 ppm. Because the carbonyl carbon and six olefinic carbons account for 4° of unsaturation, libertellenone A must possess three rings.

Further structural information was derived by analysis of ^1H COSY NMR spectral data, which illustrated the connectivity of C-1, C-2, and C-3, and the connectivity between C-11 and C-12. Interpretation of 2D HSQC and HMBC NMR data allowed all the protons and carbons to be assigned. Key HMBC correlations from 6-OH to C-6, C-5, and C-7 and from H-14 to C-8, C-13, C-7, C-17, C-15, C-12, and C-9 established the enol moiety at C-6 and showed the connectivity of C-5, C-6, C-7, C-8, C-13, C-14, C-15, and C-17, which is composed of six quaternary carbons. These data, and additional HMBC correlations from H-18 and H-19 to C-3 and C-5, from H-20 to C-10, C-1, C-5, and C-9, from H-17 to C-12 and C-14, and from H-11 to C-9 allowed the complete planar structure of **1** to be assigned as a cross-conjugated ketone of the pimarane diterpenoid class.

The relative stereochemistry of libertellenone A (**1**) was assigned by analysis of 1D ^1H NOE NMR experiments (Fig. 3) and by interpretation of proton NMR coupling constant data. The C-20 bridgehead methyl group protons showed NOE correlations with H-19, H-11β, and

Table 1. ^{1}H and ^{13}C NMR data for libertellenones A–D (**1**–**4**) in $CDCl_3$

Position	Libertellenone A (**1**)		Libertellenone B (**2**)		Libertellenone C (**3**)		Libertellenone D (**4**)	
	δ_H[a], mult (*J*)[b]	δ_C[c]	δ_H, mult (*J*)	δ_C	δ_H, mult (*J*)	δ_C	δ_H, mult (*J*)	δ_C
1	4.34 dd (9.5, 5.5)	69.3 CH	5.76 ddd (10.0, 2.0, 1.5)	128.7 CH	4.27 dd (11.0, 5.0)	69.4 CH	1.57 m[f]	11.8 CH
2α	1.74 m	29.2 CH_2	6.09 ddd (10.0, 5.0, 3.0)	128.8 CH	1.74 m	28.3 CH_2	2.30 br m	26.6 CH_2
2β							1.67 br d (13.5)	
3α	1.53 m[d]	38.4 CH_2	2.47 ddd (16.5, 3.0, 2.0)	34.6 CH_2	2.07 ddd (13.5, 13.5, 4.5)	33.9 CH_2	3.60 dd (5.0, 2.0)	69.6 CH
3β			1.82 m[e]		1.29 ddd (13.5, 3.5, 3.5)			
4		35.8 C		42.1 C		41.5 C		42.7 C
5		143.5 C		137.1 C		139.4 C		130.0 C
6		145.1 C		141.6 C		144.7 C		148.0 C
6-OH	6.75 s		6.97 s		6.81 s		6.68 s	
7		181.3 C		182.0 C		181.6 C		182.1 C
8		133.2 C		132.1 C		133.3 C		133.8 C
9		74.6 C		74.6 C		75.6 C		71.7 C
10		50.4 C		46.7 C		49.8 C		28.9 C
11α	2.01 ddd (14.0,14.0,3.5)	28.0 CH_2	1.82 m[e]	27.2 CH_2	1.95 ddd (14.0,14.0,3.5)	28.7 CH_2	1.48 m[g]	26.7 CH_2
11β	2.16 ddd (14.0,14.0,3.5)		2.09 ddd (13.5,13.5,3.0)		2.14 ddd (14.0,14.0,3.5)		1.33 ddd(13.5,13.5,3.0)	
12α	1.83 ddd (14.0,14.0,3.5)	29.6 CH_2	1.89 ddd (13.5,13.5,3.0)	29.5 CH_2	1.79 ddd (14.0,14.0,3.5)	29.4 CH_2	1.92 ddd(13.5,13.5,3.0)	29.0 CH_2
12β	1.58 m[d]		1.56 dddd (13.5,13.5,3.0, 2.0)		1.54 dddd (14.0,14.0,3.5, 2.0)		1.51 m[g]	
13		38.6 C		38.9 C		38.6 C		39.0 C
14	7.08 d (2.0)	149.1 CH	7.10 d (2.0)	149.7 CH	7.06 d (2.0)	149.0 CH	6.97 d (2.0)	148.2 CH
15	5.84 dd (17.5, 10.5)	145.4 CH	5.85 dd (17.5, 10.5)	144.9 CH	5.85 dd (17.5, 10.5)	145.4 CH	5.82 dd (17.5, 10.5)	145.0 CH
16a	5.07 dd (17.5, 1.0)	112.5 CH_2	5.08 dd (17.5, 1.0)	112.8 CH_2	5.08 dd (17.5, 1.0)	112.5 CH_2	5.07 dd (17.5, 1.0)	112.8 CH_2
16b	5.03 dd (10.5, 1.0)		5.05 dd (10.5, 1.0)		5.03 dd (10.5, 1.0)		5.03 dd (10.5, 1.0)	
17	1.11 s	23.3 CH_3	1.13 s	23.4 CH_3	1.10 s	23.1 CH_3	1.08 s	23.9 CH_3
18a	1.38 s	29.4 CH_3	4.04 d (11.0)	70.6 CH_2	4.53 d (10.5)	69.9 CH_2	4.86 d (11.5)	66.9 CH_2
18b			3.43 d (11.0)		3.08 d (10.5)		4.64 d (11.5)	
19	1.28 s	26.3 CH_3	1.23 s	23.2 CH_3	1.15 s	21.8 CH_3	1.17 s	19.5 CH_3
20α	1.19 s	22.6 CH_3	1.23 s	27.3 CH_3	1.18 s	22.8 CH_3	1.11 dd (9.5, 5.5)	21.8 CH_2
20β							0.39 dd (5.5, 5.5)	
C=O(Ac)								172.3 C
CH_3(Ac)							2.07 s	21.0 CH_3

Assignments by COSY, NOE, and multiplicity-edited gHSQC experiments.
[a] 500 MHz.
[b] Coupling constant in Hz.
[c] 125 MHz.
[d] Overlapped.
[e] Overlapped.
[f] Overlapped with H_2O peak.
[g] Overlapped.

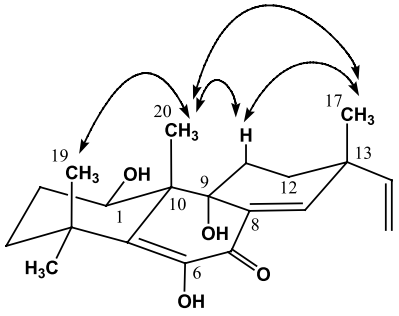

Figure 3. Three-dimensional structure of libertellenone A (**1**) with the key NOE correlations.

H-17. These correlations demonstrated that C-17, C-19, and C-20 are in axial configurations on the top face of the molecule. Proton H-11β, was assigned in the pseudo-axial position based upon NOE correlations and upon its proton coupling constants (ddd, 14.0, 14.0, 3.5 Hz), which are the characteristics of geminal and axial–axial relationships. The relative stereochemistry of the hydroxyl group at C-9 was assigned in the axial position based upon an NOE correlation between H-20 and H-11. Similarly, H-1 was assigned as axial by analysis of its coupling constants with the H-2 protons, which showed large axial–axial (9.5 Hz) and small axial–equatorial (5.5 Hz) coupling constants.

The absolute configuration of libertellenone A (**1**) was determined by application of the modified Mosher method.[13] Among the three hydroxyl groups present in **1**, only the C-1 secondary alcohol was expected to readily acylate. Acylation of **1** with *R*-(+)- and *S*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride, yielded the C-1 *S* and *R* esters in good yield. Analysis of ^{1}H NMR, ^{1}H COSY, and TOCSY NMR spectra for these Mosher esters allowed all protons to be assigned. The $\Delta\delta_{S-R}$ values for H-2, H-3, H-18, and H-19 are positive, while those of H-11, H-12, H-14, H-15, H-16, H-17, and H-20 are negative, thus the absolute configuration of the C-1 chiral center was assigned as *R* (Fig. 4). The chiral centers at C-9, C-10, and C-13 could then be assigned as *S*, *R*, and *R* based on their relative configurations as derived in the previous NOE experiments.

Libertellenone B (**2**) was obtained as pale yellow powder, which was analyzed for the molecular formula $C_{20}H_{26}O_4$ by HR-MALDI mass spectrometry (obsd

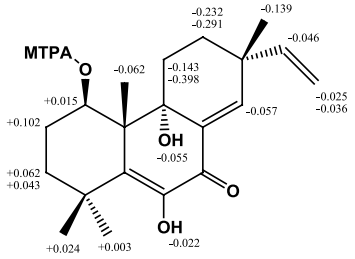

Figure 4. Delta values ($\Delta\delta_{S-R}$) in ppm for the 1*S* and 1*R* MTPA esters of libertellenone A in CD_3CN.

$[M+Na]^+$ at *m/z* 353.1727, calcd $[M+Na]^+$ 353.1729). This formula was also supported by interpretation of ^{1}H and ^{13}C NMR spectral data (Table 1). The ^{13}C NMR spectrum of **2** showed two additional sp^2 carbons, which accounted for the additional unsaturation in this diterpenoid. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1** except that one methyl group was absent, two additional sp^2 carbons were present [δ_C: 128.8; 128.7; δ_H: 6.09 (ddd, 10.0, 5.0, 3.0); 5.76 (ddd, 10.0, 2.0, 1.5)], and two new hydroxyl-bearing methylene protons were observed [δ_H: 4.04 (d, 11.0); 3.43 (d, 11.0)]. Analysis of ^{1}H COSY and HSQC NMR data showed two disconnected spin systems, one involving C-1, C-2, and C-3, and the other the connectivity of C-11 and C-12. Further analysis of HSQC and HMBC NMR spectral data allowed all the protons and their respective carbons to be assigned. The full planar structure showed that **2** was a derivative of libertellenone A in which the C-1 alcohol had undergone dehydration to generate a $\Delta^{1,2}$ olefinic bond.

As in **1**, the relative stereochemistry of **2** was determined by a combination of NMR NOE data and coupling constant analyses. Although two methyl groups were unresolved in the ^{1}H NMR spectrum of **2** (δ_H: 1.23) in $CDCl_3$, the spectrum recorded in CD_3CN allowed these two to be resolved. NOE correlations between the C-19 and C-20 methyl groups showed that they were axial and on the top face of the molecule. Additional NOE correlations between H-17 and H-11β, and between H-20 and H-11β clearly supported the assigned relative stereochemistry shown in **2**.

Libertellenone C (**3**) was obtained as a white powder, which was analyzed by HR-MALDI mass spectrometry for the molecular formula $C_{20}H_{28}O_5$ (obsd $[M+Na]^+$ at *m/z* 371.1829, calcd $[M+Na]^+$ 371.1834). The ^{1}H and ^{13}C NMR spectral data for **3** were similar to those of **1** and **2**. As in **2**, one of the methyl groups bore a hydroxyl functionality (δ_H: 4.53, d and 3.08, d), which was assigned at C-18 on the basis of heteronuclear correlations from H-19 to C-4, C-3, C-5, and C-18, and from H-18 to C-4, C-3, and C-5. Further analysis of HMBC NMR data led to the confident assignment of the full planar structure of **3** as an analogue of **2** formed by hydration of the $\Delta^{1,2}$ olefin. As in both prior libertellenones, NOE experiments showed the spatial arrangements of the methyl groups allowing the relative stereochemistry to be assigned as in structure **3**.

Libertellenone D (**4**) was isolated as pale yellow viscous oil, which was analyzed for the molecular formula $C_{22}H_{28}O_6$ by HR-MALDI mass spectrometry (obsd $[M+Na]^+$ at *m/z* 411.1778; calcd $[M+Na]^+$ 411.1784). The overall ^{1}H NMR spectral features of **4** were similar to the other libertellenones; however, **4** displayed a distinct upfield proton at 0.39 ppm (dd, 5.5, 5.5 Hz), strongly indicative of the presence of a cyclopropane ring. The existence of one additional ring was also indicated by the 9° of unsaturation inherent in the molecular formula. The presence of a sharp singlet methyl signal at 2.07 ppm, and the fact that this diterpenoid contained two additional carbons, suggested the presence of an

acetate ester. Characteristic ester IR absorptions at 1725 cm^{-1} and a downfield shifted pair of doublet protons at δ_H 4.86 and 4.64 (each d, 11.5 Hz) showed that acetylation had occurred at a primary alcohol carbon. Analysis of proton COSY and HSQC NMR spectra data revealed the connectivity of C-20, C-1, C-2, and C-3 and C-11 and C-12. Unique to **4**, the proton and carbon chemical shifts of H-20 and C-20 [δ_H: 0.39 (dd, 5.5, 5.5); 1.11 (dd, 9.5, 5.5)], [δ_C: 21.8] and H-1 and C-1 [δ_H: 1.57 (m)], [δ_C: 11.8] indicated that these protons and carbons belong to a cyclopropane ring system. Interpretation of additional HMBC correlations established the full planar structure of this diterpenoid. The cyclopropane ring system was assigned on the basis of HMBC correlations between H-20β and C-10, C-1, C-5, and C-9. As in **1**–**3**, the relative stereochemistry was established by interpretation of 1D ^1H NOE spectral data.

The absolute stereostructures of the libertellenones B–D were determined by comparison of their CD spectra.[14,15] Because the absolute stereochemistry had been determined by Mosher ester analysis for libertellenone A, comparison CD spectra were obtained for all four diterpenoids. Libertellenone C (**3**) showed a virtually identical CD spectrum to that of libertellenone A (**1**) with both demonstrating a clear negative Cotton effect between 350 and 360 nm. Thus, the absolute configuration of **3** was assigned as 1*R*, 4*R*, 9*S*, 10*R*, and 13*R*. The CD spectra of **2** and **4** displayed different features from that of **1** at shorter wavelengths (less than 300 nm), apparently due to modifications in the A ring. However, the negative Cotton effect at 340–360 nm, which is derived from the π–π^* transition of the cross-conjugated system, was consistent and established the absolute stereochemistry of **2** as 4*R*, 9*S*, 10*S*, and 13*R* and that of **4** as 1*R*, 3*R*, 4*R*, 9*S*, 10*S*, and 13*R*.

Numerous pimarane diterpenoids have been isolated from plants and from plant pathogenic fungi.[16] The novelty of the libertellenones A–D lies in the high degree of unsaturation inherent in the cross-conjugated ketone functionality. A similar cross-conjugated ketone system has been reported in the abietane diterpenoids,[17] however, libertellenone D is most closely related to myrocin A, a cross-conjugated, cyclopropane-containing pimarane that, interestingly, is produced by a marine fungal strain.[18]

Induction of the biosynthesis of **1**–**4** was reliably observed in numerous replicate co-culture experiments. In seven out of seven experiments, diterpenoid production was not observed when either the fungus *Libertella* sp. or the bacterium CNJ-328 were cultured independently. In these same 1 L experiments, the addition of a small volume (1 mL) of the bacterium CNJ-328 culture to the fungal culture caused rapid (within 24 h) induction of the biosynthesis of diterpenoids **1**–**4**. In an effort to determine the factor(s) that trigger the biosynthesis of these diterpenoids, several experiments were performed. In these experiments, neither the addition of dead bacterial cells (autoclaved or heat killed at 70 °C for 30 min) nor the cell-free supernatant or ethyl acetate (EtOAc) extract of a viable bacterial culture induced diterpene biosynthesis. Thus, there was no evidence for cross-Kingdom signaling molecules, a phenomenon recently reported between bacteria and fungi,[19] and it would appear that induction is controlled by cell–cell interactions.

To explore if other bacteria or fungi would induce production when co-cultured with either *Libertella* or the α-proteobacterium CNJ-328, we conducted various combinations of co-culture experiments with these microorganisms against a variety of fungi and bacteria. In no cases were the libertellenones produced. Because it was not possible to induce diterpene production, the fungus could not be unequivocally identified as the biosynthetic source of these compounds. However, given that terpenoids are commonly produced by fungi and plants,[20] that they are rarely the products of bacterial metabolism,[21] and that pimarane diterpenes have never been reported from bacteria, there is a high probability that these compounds are produced by the fungus. It is noteworthy, however, that in a prior study we isolated a series of new verrucosane diterpenes from a marine bacterium.[22] Thus, without additional experimental evidence, a bacterial origin for these compounds cannot be rigorously ruled out.

It is tempting to hypothesize that the induction of diterpenoid biosynthesis is in response to microbial competition. It has been speculated that the rationale for the abundant synthesis of microbial antibiotics reflects the need in nature for strategies to enhance survival.[23] Because libertellenone production was induced in co-culture, in what can be considered a simulated competitive environment, we expected to see evidence of antibacterial activity in these compounds. However, that was not the case. None of the libertellenones showed significant activity against the bacterium CNJ-328 even at elevated concentrations. The diterpenoids were also devoid of activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. Libertellenones A–D showed only weak activity (MIC > 160 μg/mL) against *Candida albicans* (wild type and amphotericin B-resistant strains). To examine alternative modes of bioactivity, we evaluated the libertellenones for cytotoxicity against the HCT-116 human colon carcinoma cancer cell line. Libertellenone D (**4**) demonstrated potent cytotoxicity (IC_{50} = 0.76 μM), while libertellenones A–C were much less cytotoxic (IC_{50} = 15, 15, and 53 μM, respectively). Thus, the cyclopropane ring in **4** appears to be an important structural feature associated with the biological activity of this compound.

3. Experimental

3.1. General experimental procedures

^1H, ^{13}C, and 2D NMR spectral data were obtained on a Varian Inova 500 MHz and a Varian Inova 300 MHz NMR spectrometers. UV spectra were recorded in a Perkin-Elmer Lamda 19 spectrometer with a path length

of 1 cm. IR spectra were acquired in a Perkin-Elmer 1600 FT-IR spectrometer. Optical rotations were measured using a Rudolph Research Autopol III polarimeter with a 10-cm cell. High resolution MALDI-FTMS data were collected on an IonSpec Ultima mass spectrometer at the Scripps Research Institute, La Jolla. Low resolution LC/MS data were acquired using a Hewlett-Packard series 1100 LC/MS system with a reversed-phase C18 column (Agilent, 4.6 mm × 100 mm, 5 μm) at the flow rate of 0.7 mL/min. LC/MS analysis used a CH_3CN/H_2O gradient solvent system beginning with 10% aqueous CH_3CN and ending at 100% CH_3CN at 30 min. The CD spectra were obtained on a Jasco 810 spectropolarimeter with a path length of 1 cm.

3.2. The marine fungus *Libertella* (CNL-523) and the marine α-proteobacterium (CNJ-328)

The marine fungus (CNL-523) was isolated from an ascidian collected in the Bahamas in 1996 and identified as a *Libertella* sp., based on morphological characteristics, by the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl). The marine bacterium (CNJ-328) was isolated as a contaminant from a fungal culture and is the same strain that was found to induce production of the antibiotic pestalone in fungal strain CNL-365.[11] NCBI blastn analysis of the partial 16S rRNA gene of CNJ-328 (deposited with GenBank as Accession No. AY989809) indicates that this strain shows 100% identity to an unidentified α-proteobacterium (AF493974) cultivated from non-axenic cultures of the cyanobacterium *Prochlorococcus marinus*. Interestingly, CNJ-328 also shows close identity (99%) to uncultured α-proteobacteria (AJ294358 and AY701438) from the dinoflagellates *Alexandrium* sp. and *Gymnodinium catenatum* (respectively) and 98% identity to an algicidal bacterium from the family Rhodospirillaceae (AB180392) and an endocytic bacterium (AF262750) isolated from the dinoflagellate *Noctiluca scintillans*. Thus, CNJ-328 and many of its relatives are commonly found in association with other microorganisms. The most closely related bacterium that has been identified is *Thalassospira lucentensis* (98% identity, AF358664), and thus CNJ-328 may represent a new *Thalassospira* sp.

3.3. Fermentation and co-culture experiment

The fungus was cultured in multiple 2.8-L Fernbach flasks each containing 1 L of the medium YPM (4 g mannitol, 2 g yeast extract, 2 g peptone, and 1 L seawater) at 27 °C with shaking at 215 rpm. The bacterium was cultivated in the same manner as the fungus but in a total volume of 1 L. The co-culture production fermentation was initiated by inoculating 1 mL of a 3-day bacterial culture into each 1 L of the 3-day-old fungal cultures. The co-culture was incubated for two additional days before extraction. Exhaustive time-course studies were performed on pure cultures of the fungus and bacterium and various mixtures of the two, all of which were monitored by LC/MS for libertellenone production. For LC/MS analysis, 25 mL samples were extracted with EtOAc.

3.4. Extraction

At the end of the fermentation period, Amberlite XAD-7 resin (20 g/L) was added to each 1-L co-culture to adsorb excreted organic substances. The culture and resin were shaken at 215 rpm for two additional hours. The resin and cell mass were filtered through cheesecloth and washed with DI water to remove salts. The resin, cell mass, and the cheesecloth were soaked in acetone and shaken at 215 rpm for 1 h. The acetone extract was dried in vacuo to give 52 g of crude material from a 40-L culture.

3.5. Isolation of libertellenones A–D

The extract was subjected to a silica gel column chromatography (column diameter 2.5 cm, height 45 cm, 200–425 mesh). The fractions from the 5:3 mixture of EtOAc and isooctane and 100% EtOAc solvents yielded 2.60 g of a terpenoid mixture by NMR and TLC analysis. CH_3CN soluble materials (1.97 g) were fractionated by reversed-phase HPLC with 42% aqueous CH_3CN (Waters Prep LC 4000 system, Waters preparative column C18 60 Å, 25 mm × 200 mm, 10 mL/min, UV detection at 210 nm). Libertellenone C (t_R = 27 min) was finally isolated from the HPLC fraction (t_R = 11 min) by further reversed-phase HPLC (Dynamax semi-preparative column C18 60 Å, 10 mm × 250 mm, 2 mL/min, refractive index detection) with 37% aqueous CH_3CN (6.3 mg yield). Libertellenone A (20 mg), B (25 mg), and D (3.7 mg) were purified from the first HPLC fraction (t_R = 24 min) by normal-phase HPLC (Lichrospher semi-preparative column Si gel, 10 mm × 250 mm, 2 mL/min, refractive index detection) with 50% isooctane in EtOAc. Libertellenones A, B, and D were eluted at 12, 19, and 23 min, respectively, in the final HPLC.

3.5.1. Libertellenone A (1). White powder; $[\alpha]_D$ −96.8° (*c* 0.133, CH_3CN); UV (CH_3CN) λ_{max} (log ε) 322 (3.67), 266 (3.45), 216 (3.55) nm; CD (CH_3CN) (Δε) 353 (−50.2), 311 (+9.1); IR (neat, $CHCl_3$) υ_{max} 3375, 2970, 1666, 1378, 1001 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), HR-MALDI $[M+Na]^+$ *m/z* 355.1885.

3.5.2. Libertellenone B (2). Pale yellow powder; $[\alpha]_D$ −64.7° (*c* 0.973, CH_3CN); UV (CH_3CN) λ_{max} (log ε) 317 (3.80), 263 (3.57), 216 (3.62) nm; CD (CH_3CN) (Δε) 342 (−21.0), 258 (+11.6); IR (neat, $CHCl_3$) υ_{max} 3395, 2963, 1653, 1374, 1039 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), HR-MALDI $[M+Na]^+$ *m/z* 353.1727.

3.5.3. Libertellenone C (3). White powder; $[\alpha]_D$ −84.1° (*c* 0.387, CH_3CN); UV (CH_3CN) λ_{max} (log ε) 321 (3.70), 269 (3.48), 216 (3.54) nm; CD (CH_3CN) (Δε) 355 (−55.2), 312 (+17.8); IR (neat, $CHCl_3$) υ_{max} 3375, 2918, 1650, 1359, 1011 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), HR-MALDI $[M+Na]^+$ *m/z* 371.1829.

3.5.4. Libertellenone D (4). Viscous oil; $[\alpha]_D$ −145.1° (*c* 0.247, CH_3CN); UV (CH_3CN) λ_{max} (log ε) 327 (3.73),

267 (3.71), 216 (3.83) nm; CD (CH_3CN) ($\Delta\varepsilon$) 360 (−56.0), 319 (+28.8); IR (neat, $CHCl_3$) ν_{max} 3388, 2943, 1725, 1663, 1393, 1246, 1030 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), HR-MALDI $[M+Na]^+$ *m/z* 411.1778.

3.6. Preparation of *R*- and *S*-MTPA ester derivatives of libertellenone A (1)

Replicate 1.8 mg (5.4 μmol) samples of libertellenone A (**1**) were prepared for separate Mosher acylation. The samples were dried under high vacuum and dissolved in 1.5 mL of freshly distilled dichloromethane containing a catalytic amount of triethylamine in two 8-mL vials. To the two vials were added a small dry crystal of dimethylaminopyridine (DMAP), and after stirring for 1 h, 20 μL of (*R*)-MTPA chloride solution (5.36 μmol/μL) and (*S*)-MTPA chloride were added. After incubating the solutions at room temperature for 1 h, 0.5 mL of freshly distilled pyridine was added. The reaction with (*R*)-MTPA chloride was complete after 2 h, but the acylation with (*S*)-MTPA chloride was sluggish and required 4 days to obtain an 80% yield of the product. Both (*R*)- and (*S*)-MTPA esters were purified by reversed-phase HPLC (Hewlett-Packard series 1050, Alltech, Altima C18, 10.0 mm × 250 mm, 5 μm, 2.0 mL/min, UV detection at 210 nm) using a gradient solvent system (0–10 min. 20% aqueous CH_3CN; 10–50 min, 20–100% aqueous CH_3CN; 50–60 min, 100% CH_3CN). The *R*- and *S*-ester derivatives were eluted at 55.2 min. The molecular formulae for the *R*- and *S*-esters were confirmed as $C_{30}H_{35}F_3O_6$ by API-ES LC/MS methods ($[M+H]^+$ *m/z* 549 and $[M+Na]^+$ *m/z* 571).

Acknowledgments

This work is a result of generous research support provided by the National Cancer Institute under Grants CA 44848 and CA 67775. We thank Sara Kelly for performing the antibacterial, antifungal, and cytotoxic bioassays.

Supplementary data

^1H and ^{13}C NMR spectra of libertellenones A and B (**1**, **2**) and ^1H NMR spectra of libertellenones C and D (**3**, **4**). This material is available via the Internet at http://www.sciencedirect.com and doi:10.1016/j.bmc.2005.05.068

References and notes

1. Brock, T. D. *Principles of Microbial Ecology*; Prentice Hall: Englewood Cliffs, NJ, 1966, pp 127–134.
2. Alexander, M. *Microbial Ecology*; Wiley: New York, 1971, pp 300–326.
3. Lai, P.; Bruehl, G. W. *Phytopathology* **1968**, *58*, 562.
4. Long, R.; Azam, F. *Appl. Env. Microbiol* **2001**, *67*, 4975.
5. Mearns-Spragg, A.; Bregu, M.; Boyd, K. G.; Burgess, J. G. *Lett. Appl. Microbiol* **1998**, *27*, 142.
6. Burgess, J. G.; Jordan, E. M.; Bregu, M.; Mearns-Spragg, A.; Boyd, K. G. *J. Biotechnol.* **1999**, *70*, 27.
7. Ueda, K.; Kwai, S.; Ogawa, H.-O.; Kiyama, A.; Kubota, T.; Kwanobe, H.; Beppu, T. *J. Antibiot.* **2000**, *53*, 979.
8. Sonnebichler, J.; Dietrich, J.; Peipp, H. *Biol. Chem. Hoppe-Seyler* **1994**, *375*, 71.
9. Degenkolb, T.; Heinze, S.; Schlegel, B.; Strobel, G.; Grafe, U. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 883.
10. Slattery, M.; Rajbhandari, J.; Wesson, K. *Microb. Ecol.* **2001**, *41*, 90.
11. Cueto, M.; Jensen, P. R.; Kauffman, C.; Fenical, W.; Lobkovsky, E.; Clardy, J. *J. Nat. Prod.* **2001**, *64*, 1444.
12. Garbaye, J. *New Phytol.* **1994**, *128*, 197.
13. Seco, J. M.; Quinoa, E.; Riguera, R. *Tetrahedron: Asymmetry* **2001**, *12*, 2915.
14. Nakanishi, K.; Berova, N.; Woody, R. W. *Circular Dichroism: Principles and Applications*; VCH publisher: New York, 1994.
15. Watanabe, K.; Sekine, M.; Iguchi, K. *J. Nat. Prod.* **2003**, *66*, 1434.
16. Evidente, A.; Sparapano, L.; Andolfi, A.; Bruno, G.; Motta, A. *Aust. J. Chem.* **2003**, *56*, 615.
17. Mei, S.-X.; Jiang, B.; Niu, X.-M.; Li, M.-L.; Yang, H.; Na, Z.; Lin, Z.-W.; Li, C.-M.; Sun, H.-D. *J. Nat. Prod.* **2002**, *65*, 633.
18. Klemke, C.; Kehraus, S.; Wright, A. D.; Konig, G. M. *J. Nat. Prod.* **2004**, *67*, 1058.
19. Wang, L.-H.; He, Y.; Gao, Y.; Wu, J. E.; Dong, Y.-H.; He, C.; Wang, S. X.; Weng, L.-X.; Xu, J.-L.; Tay, L.; Fang, R. X.; Zhang, L.-H. *Mol. Microbiol.* **2004**, *51*, 903.
20. Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II*; Academic Press: London, 1983, pp 272–302.
21. Hefter, J.; Richnow, H. H.; Fischer, U.; Trendel, J. M.; Michaelis, W. *J. Gen. Microbiol.* **1993**, *139*, 2757.
22. Spyere, A.; Rowley, D. C.; Jensen, P. R.; Fenical, W. *J. Nat. Prod.* **2003**, *66*, 818.
23. Walsh, C. *Antibiotics: Actions Origins Resistance*; ASM Press: Washington, DC, 2003, pp 5–6.